Universidade do Minho
Escola de Ciências

Mariana Isabel Cunha Nogueira Almeida Monteiro

Plant extracts: isolation, characterization, encapsulation in lipid nanosystems and cytotoxicity in AGS cells

Outubro 2018

Plant extracts: isolation, characterization encapsulation in lipid nanosystems and cytotoxicity in AGS cells
UMinho | 2018 Mariana Isabel Cunha Nogueira Almeida Monteiro

Universidade do Minho

Escola de Ciências

Mariana Isabel Cunha Nogueira Almeida Monteiro

Plant extracts: isolation, characterization, encapsulation in lipid nanosystems and cytotoxicity in AGS cells

Dissertação de Mestrado

Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação de

Professor Doutor A. Gil Fortes

Professora Doutora Elisabete M. S. Castanheira Coutinho

Outubro de 2018

DECLARAÇÃO

Nome: Mariana Isabel Cunha Nogueira Almeida Monteiro

Endereço eletrónico: marianaicnamonteiro@gmail.com Telefone: +351 916444912

Bilhete de Identidade/Cartão do Cidadão: 14652742 9ZY2

Título da dissertação: Plant extracts: isolation, characterization, encapsulation in lipid nanosystems and cytotoxicity in AGS cells

Orientadores:

Professor Doutor A. Gil Fortes

Professora Doutora Elisabete M. S. Castanheira Coutinho

Ano de conclusão: 2018

Mestrado em Bioquímica Aplicada

Universidade do Minho, 31/10/2018

Assinatura 

Acknowledgments

This work could not be carried out without the participation of other stakeholders that directly or indirectly contributed to its success.

First of all, I would like to thank Professor António Gil Fortes and Professor Maria Sameiro Torres Gonçalves for the opportunity to participate in this new project. Also thank you for the knowledge, incentive, experience and availability, demonstrated during the year.

To Professor Eisabete Coutinho for the opportunity that she gave me to learn and gain experience in the field of physics and for all availability in the doubts and corrections of this thesis.

To Professor David Pereira for the opportunity to work in the laboratory at the Faculty of Pharmacy at the University of Porto.

I would also like to thank the entire Department of Chemistry/Chemistry Centre of the University of Minho, which provided me with all the means for such experiments, and also the Laboratory of Pharmacognosy of the Department of Chemical Sciences of the Faculty of Pharmacy/REQUIMTE of the University of Porto and the Department of Physics/Physics Centre of the University of Minho. Thanks are due to Fundação para a Ciência e Tecnologia (FCT-Portugal) and FEDER through the project PTDC/ASP-AGR/30154/2017.

To Dr. Elisa Pinto and to Vania Azevedo for the professionalism shown to the requests for the NMR and mass spectra.

To my laboratory colleagues, Rita Araújo and Rui Sousa for the company, joke, patience, wisdom, for helping me whenever I needed and for washing my laboratory material when I do not have time.

To the "Grupo da Marmita" that always supported me, especially at times of greater stress. Thanks for all those relaxing moments on the red sofas.To my girls from the Faculty of Porto, for all the support and for all the lunches and dinners.To the “Besouros”, for being my 2nd Family, for supporting me in all my decisions; for putting up with me in my state "Monica", for all the coffees that made me distract. Thank you! And last but not least to my family for always believing in me and always telling me that I will get far.

Abstract

Plants contain essential products for the maintenance of the cell (lipids, proteins, carbohydrates and nucleic acids) and by-products that come from several biosynthetic pathways. These by-products are named phytochemicals and include phenols, glycosides, alkaloids and terpenoids. Phytochemicals possess interesting potential biological activities, however, some are extremely unstable, which could be a handicap in potential application of these plant extracts. In this work, *Phytolacca americana L.*, *Tagete patula L.*, *Cotoneaster horizontalis Decne*, *Rosmarinus officinalis L.* and *Aloe barbadensis Miller* were chosen for extraction and characterisation of their phytochemicals and also to evaluate their anticancer potential using non-encapsulated and lipid encapsulated extracts. Various spectroscopic and chromatographic techniques have been used for extract composition analysis, namely ^{1}H- and ^{13}C-NMR, HPLC-DAD, HRMS, IR, UV-Vis. Saponins, terpenes, phenolics, carotenoids and anthraquinone type compounds are amongst the phytochemicals present in the extracts. In the case of rosemary, it was possible to isolate and fully identify a single compound, verbenone. All the extracts/fractions from these species have shown toxicity against human adenocarcinoma stomach cells and only the toxicity of extract (after hydrolysis) from pokeweed and verbenone are related to processes leading to cell necrosis. The encapsulation of extract obtained before hydrolysis from pokeweed and of one fraction collected from French marigold have shown high efficiencies up to 98%; however the encapsulation of extract obtained from pokeweed after hydrolysis, has shown a lower efficiency. Overall, the results obtained are interesting and encouraging for further studies.

Keywords: phytochemicals; extraction techniques; biological activity; nanoencapsulation in lipid systems;

Resumo

As plantas contêm produtos essenciais para a manutenção da célula (lípidos, proteínas, carboidratos e ácidos nucleicos) e subprodutos que vêm de várias vias biossintéticas. Esses subprodutos são denominados fitoquímicos e incluem fenóis, glicosídeos, alcaloides e terpenoides. Os fitoquímicos possuem interessantes atividades biológicas; no entanto, alguns são extremamente instáveis, o que poderia ser uma desvantagem na potencial aplicação destes extratos vegetais. Neste trabalho, *Phytolacca americana L.*, *Tagete patula L.*, *Cotoneaster horizontalis Decne*, *Rosmarinus officinalis L.* e *Aloe barbadensis Miller* foram escolhidas para a extração e caracterização dos seus fitoquímicos e também para avaliar o seu potencial anticancerígeno utilizando extratos encapsulados e não encapsulados. Diversas técnicas espectroscópicas e cromatográficas foram utilizadas para a determinação da composição do extrato, principalmente RMN de ^{1}H e ^{13}C, HPLC-DAD, HRMS, IV, UV-Vis. Saponinas, terpenos, compostos fenólicos, carotenoides e antraquinonas estão entre os fitoquímicos presentes nos extratos. No caso do alecrim, foi possível isolar e identificar completamente um único composto, a verbenona. Todos os extratos/frações destas espécies mostraram toxicidade contra células de adenocarcinoma do estômago humano e apenas a toxicidade do extrato (após hidrólise) da uva-de cão e a verbenona estão relacionadas a processos que levam à necrose celular. O encapsulamento do extrato obtido antes da hidrólise da uva-de-cão e de uma fração obtida do cravo da França apresentaram elevadas eficiências com valores até 98%; no entanto, o encapsulamento do extrato obtido da uva-de-cão após hidrólise, mostrou uma baixa eficiência. No geral, os resultados obtidos são interessantes e encorajadores para estudos futuros.

Palavras-chave: fitoquímicos; técnicas de extração; atividade biológica; nanoencapsulamento em sistemas lipídicos;

Table of contents

Index of illustrations

Index of tables

Index of schemes

List of abbreviations

δ	chemical shift
λ	wavelength
ε	molar absorption coefficient
ν_{max}	maximum wavenumber (expressed in cm^{-1})
Abs	absortion
AGS	adenocarcinoma gastric stomach
APCI	atmospheric pressure chemical
CPS	counts per second
d	doublet
DAD	diode array detector
DCM	dichloromethane
dd	double doublet
DMEM	Dulbecco's modification of Eagle medium
DMSO	dimethyl sulfoxide
dt	doublet of triplet
EOs	essential oils
ESI	electrospray ionization
FTIR	fourier-transform infrared
GC	gas chromatoraphy
GLC	gas-lquid chromatography
HMBC	heteronuclear multiple bond correlation
HMQC	hetronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
Hz	Hertz
IR	infrared
J	Coupling constant
LC	liquid chromatography
LDH	lactate dehydrogenase
LPs	liposomes

LRMS	low resolution mass spectrometry
m	multiplet
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
ppm	parts per million
q	quaternary
Rf	retention factor
RT	retention time
t	triplet
td	triplet of doublet
TLC	thin-layer chromatography
TOF	time of fight
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet
UV-Vis	ultraviolet-visible

Communication

M. Monteiro, E. M. S. Castanheira, M. S. T. Gonçalves, A. Gil Fortes "Essential oils: extraction and encapsulation in lipid based nanosystems", poster communication (P19), *V* Encontro em Técnicas de Caracterização e Análise Química, Braga, Portugal, 4th may 2018.

Chapter 1: Introduction

1.1 Plants

Plants have a high phytochemical diversity in which many of them possess interesting physicochemical properties and potential biological activities. Plants contain several types of nutrients such as carbohydrates, proteins, lipids, minerals and vitamins, as well as non-nutritive substances, such as phenolic compounds, glycosides, alkaloids and terpenoids. These substances are called phytochemicals (Russo, 2007). Phytochemicals are secondary metabolic products of plants that play an important role in the pharmaceutical, food, cosmetic and agricultural areas due to their biological properties (Crozier *et al.*, 2009). The function of these products is to protect plant cells from pollution, stress, drought, UV exposure and pathogenic attack (Kennedy *et al.*, 2011). These compounds have biological properties such as antioxidant, antimicrobial, cytotoxic and anticancer (Rao, 2003). With such biological properties, phytochemicals have great utility in human health and therefore more than 4000 phytochemicals have been catalogued (Kurmukov, 2013). In this way, the components of the plants can be extracted and characterized using different techniques and used in the several areas already mentioned above (Yalavarthi *et al.*, 2013).

1.2 Classification of phytochemicals

1.2.1 Essential oils

Natural hydrocarbons can be divided in three main classes: aliphatic, aromatic and terpenic. Essential oils (EOs) are complex mixtures of by-products consisting essentially of phenylpropenes and terpenes with low boiling point (Greathead, 2003). The terpenic compounds, major constituents in many EOs, are usually monoterpenic (**1** and **2**), sequisterpenic **3**, diterpenic **4** and triterpenic **5**, according to the number of isoprene **6** units, 2, 3, 4 or 6 respectively. EOs are mainly produced by glandular trichomes and are synthesized for the purpose of protecting plants from various pathogens (El-baz *et al.*, 2015). These complex mixtures have some peculiar characteristics like hydrophobicity, volatility and odour and can be found in various parts of the plant such as flowers, seeds, leaves, bark, wood, fruits and roots (Negi,

2012). Menthol **1** and limonene **2** are two examples of EOs (Eze, 2016). Terpenoids are compounds derived from isoprene, most of them are cyclic and have various functional groups. Two examples of terpenoids are menthol **1** and linalool **7** (Harbone *et al.*, 1991). All the compounds mentioned above are presented in Figure 1.

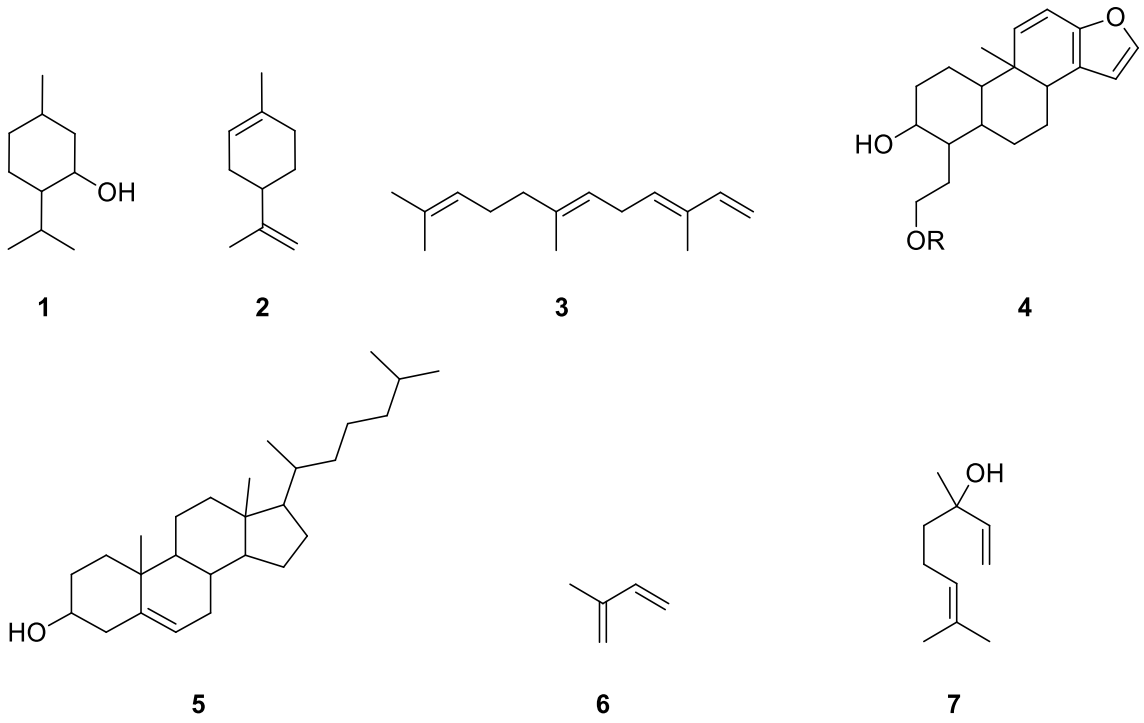


Figure 1. Structures of some EOs, terpenes and terpenoids: menthol **1**, limonene **2**, sequiterpene (farnesene) **3**, diterpenoid (kahweol) **4**, triterpenoid (lanosterol) **5**, isoprene (terpene unit) **6**, terpenoid (linalool) **7**.

1.2.2 Phenolic compounds

Phenolic compounds are a major class of constituents present in plants, which are structurally diverse. These compounds are formed by one or more aromatic rings having one or more hydroxyl groups. This class is divided into four subclasses: flavonoids, phenolic acids, tannins and quinones (Dai *et al.*, 2010). Flavonoids consist of a six-carbon ring conjugated with a benzene ring. This six carbons ring is a pyrone **8** or a hydroderivative **9**. Most flavonoids occur naturally associated with a sugar in the conjugated form (Cushnie *et al.*, 2005). Phenolic acids are phenols, which have a

carboxylic acid as the functional group. The most abundant compounds in this subclass are the hydroxycinnamic acid **10** and *p*-hydroxybenzoic acid **11** (Balasundram *et al.*, 2006) (Figure 2).

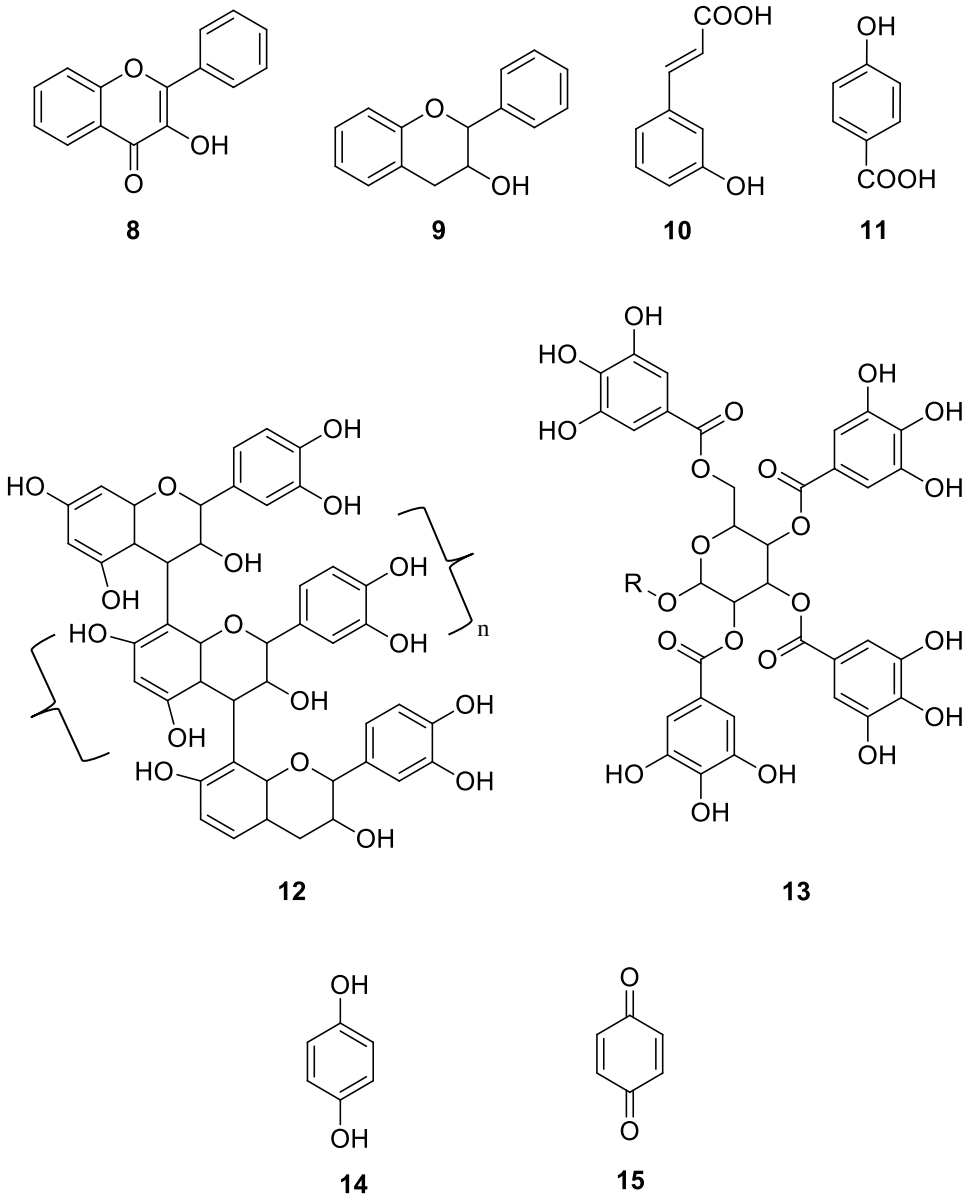


Figure 2. Examples of structures of the four subclasses of phenolic compounds: flavonoids (Flavanol, 3-hydroxy-2-phenyl-chromen-4-one **3**; flavan-3-ol, 2-phenyl-chromen-3-ol **4**), phenolic acids (*E*-3-hydroxycinnamic acid **5**; *p*-hydroxybenzoic acid **6**), tannins (condensed tannins **7**; hydrolysable tannins **8**) and quinones (hydroquinone **9**; quinone **10**).

Tannins are high molecular weight phenolic compounds, capable of forming reversible and irreversible complexes with proteins, polysaccharides, alkaloids, nucleic acids and minerals. Tannins consist in two types of different compounds: condensed tannins **12** and hydrolysable tannins **13** (Dolara *et al.*, 2005). Quinones are aromatic rings with two ketone groups. These compounds are ubiquitous in nature and are characteristically reactive. The switch between benzene-1,4-diol (or hydroquinone) **14** and diketone (or quinone) **15** occurs easily through oxidation and reduction reactions (Cowan, 1999) (Figure 2).

1.2.3 Glycosides

Glycosides consist principally of two parts: glycone (for example a sugar) and aglycone. This class of compounds is also divided into subclasses: cardiac glycosides **16**, cyanogenic glycosides **17**, glucosinolates **18** and saponins **19**. The difference between these 4 subclasses is the aglycone part (Lacaille-Dubois *et al.*, 2000). These four subclasses are presented in Figure 3.

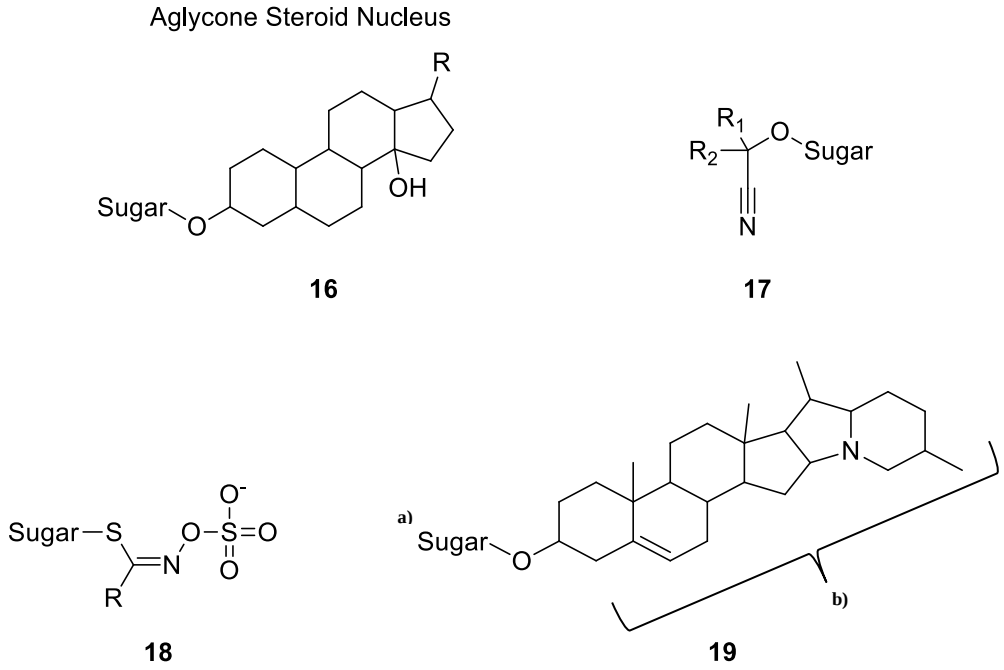
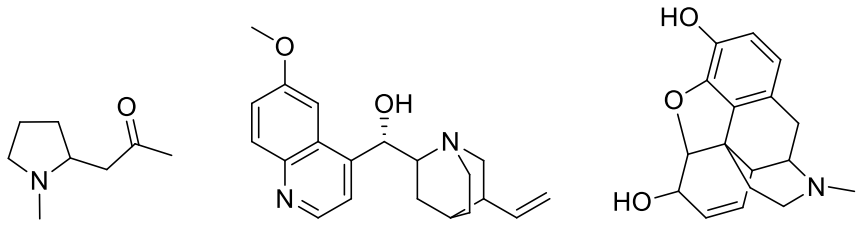


Figure 3. Structures of the four subclasses of glycosides: cardiac glycoside **16**, cyanogenic glycoside **17**, glucosinolate **18** and saponin glycoside **19**. a) represents the hydrophilic glycone and b) represents the hydrophobic aglycone.

1.2.4 Alkaloids

Alkaloids are compounds that are abundant in nature. These compounds are heterocyclic and contain nitrogen. Based on the type of heterocyclic ring, alkaloids can be divided into subclasses. Some of these subclasses are pyrrolidine alkaloids **20**, quinoline alkaloids **21** and isoquinoline alkaloids **22** (Saxena *et al.*, 2013). These compounds are presented in Figure 4.



20 **21** **22**

Figure 4. Structures of some types of alkaloids: pyrrolidine alkaloids (hygrine) **20**, quinolone alkaloids (quinine) **21**, isoquinoline alkaloids (morphine) **22**.

1.3 Plant species

The species chosen for this study are *Phytolacca americana L.*, *Tagete patula L.*, *Cotoneaster horizontalis Decne*, *Rosmarinus officinalis L.* and *Aloe barbadensis Miller*. The choice was made based on the criteria of being less studied and distributed in north region of Portugal.

1.3.1 *Phytolacca americana L.*

The pokeweed (*Phytolacca americana L.*) is a member of the *Phytolaccaceae* family, order *Centrospermae* (or *Caryophyllales*) and suborder *Chenopodiineae*. This species is native from North America, but, it has become an invasive alien plant common across Europe. In Portugal, this species grow in Minho, Trás-os-Montes, Douro Litoral, Beira, Estremadura, Ribatejo, Alto Alentejo, Algarve, Azores

archipelago and Madeira archipelago ("*Phytolacca americana* (tintureira)", 2015). *P. americana L.* is a perennial herbaceous which grows up to 3.7 m in height, produces white or purplish perfect flowers in racemes up to 0.15 m long, oblong to ovate-lanceolate leaves up to 0.15 m long and black-purple berries (Figure 5). Preparations of *P. Americana L.* have been used extensively as traditional medicines for the treatment of many disorders because of their anti-inflammatory, antimicrobial, anticancer, stimulant effects, among others. Pokeberries were reported to be toxic to various animals, despite the fact that many wild birds are known to consume these fruits (Petit-Paly *et al.*,1994).

Figure 5. Morphology of *Phytolacca americana L.*

The main constituents of pokeweed are betalains, lignanes, proteins and saponins (Figure 6). Betalains are water soluble pigments, which could be betacyanins (red-violet) **23** or betaxanthins (yellow-orange) **24** (Jerz *et al.*, 2008). Lignanes could be isolated from the seeds as neolignanes (americanol A) **25** (Zealand, 1975). This species constitute one of the best sources of ribosome-inactivating proteins (PAP – pokeweed antiviral protein) which present antiviral activity (Iglesias *et al.*, 2016). *P. americana L.* has high levels of saponins showing various pharmacological activities including anti-inflammatory (Petit-Paly *et al.*, 1994), antioxidant, anticancer (J. Das *et al.*, 2014), insecticidal (Ding *et al.*, 2013), anthelmintic, molluscicidal (Zealand, 1975) and antimicrobial. Saponins are a vast group of glycosides, which can dissolve in water to form colloidal solutions that foam upon shaking. This group of compounds can be classified into two subgroups based on the nature of their aglycone skeleton. The first group consists of the steroidal saponins, which consist of a steroidal aglycone (α-

spinasterol **26**). The second group are based on triterpenoid saponins, which consist of a triterpenoid aglycone, with a C30 skeleton, comprising a pentacyclic structure (oleanoic acid **27**) (Sparg *et al.*, 2004; Woo, 1974).

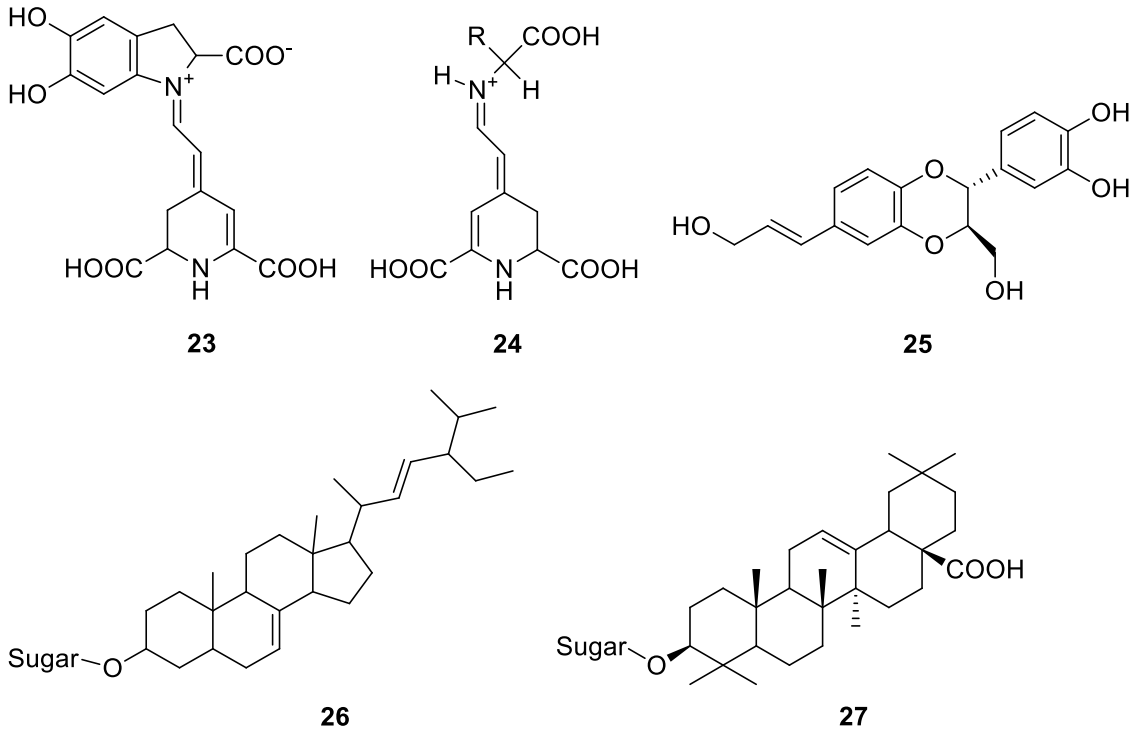


Figure 6. Structures of the main constituents of pokeweed: betalains (betacyanin **23** and betaxanthin **24)**, lignanes (americanol A **25**) and saponins: steroidal saponins (α-spinasterol **26**) and triterpenoid saponins (oleanoic acid **27**).

1.3.2 *Tagete patula L.*

Tagete patula L. (French marigold) belongs to the family *Compositae* and *Tagetes* genus. This species is native from Mexico and widely disseminated worldwide. French marigold is an annual plant with 20-30 cm height; its flowers have varied coloration (yellow, orange or red petals). *T. patula L.* is easily cultured and propagated producing flowers and seeds throughout the year, with high germinator rates (Figure 7) (Politi *et al.*, 2012).

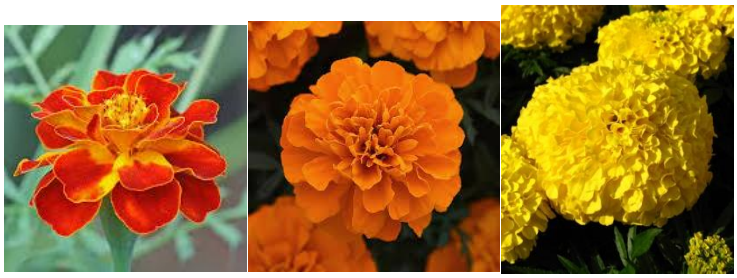


Figure 7. Morphology of *Tagete patula L.*

This plant is wdely known for its phytochemical and medicinal properties. French marigold is traditionally used to treat cough, colic, constipation, diarrhea, rheumatism and eye problems. The flowers of *T. patula L.* are edible and used in refreshing drinks. The plant is also known to possess antimicrobial, antiseptic, blood purifying and diuretic properties (Jabeen *et al.*, 2016).

T. patula L. is recognized as a source of carotenoids, phenolic compounds (flavonoids and phenolic acids), tiophenes, terpenoids and essential oils. Marigold flower petals are used as a natural coloring agent in food and as a medicine for inflamatory diseases, cardiovascular disorder, stroke and UV radiation induced skin damage. Lutein **28** ($C_{40}H_{56}O_2$; 3,30-dihydroxy-β-carotene), is a yellow plant pigment of the carotenoid family, ocurring in many flower petals. This compound is well-known for its antioxidant potential in promoting the health of eyes and skin and in reducing the risk of age-related macular degeneration, cataracts, cancer and cardiovascular disease (Bhattacharyya *et al.*, 2010). This carotenoid is present at extremely high concentration in French marigold flowers, which is commercially utilized in making dietary supplements and carotenoid-enriched foods and beverages (Abdel-Aal *et al.*, 2015). This compound was also encapsulated, which allow the solubilization of lutein in the aqueous medium and increased its stability at different temperatures (Brum *et al.*, 2017). This species is also a source of comercially important helenien and xanthophyll. Helenien is used in pharmaceuticals especially in eye care formulations. Xanthophyll is used as a direct and indirect colouring *(Garg et al.*, 1999). Polar extracts of *T. patula L.* showed presence of phenolic compounds (flavonoids and phenolics acids) in which patuletin **29** is one of the major flavonoids. This compound (3,5,7,3',4'-pentahydroxy-6-methoxy flavone) was first isolated by Rao and Seshadri in 1941 from the petals of *T.*

patula L.. Patuletin is known to possess various biological activties, which include radical scavenging, anti-inflammatory, antimicrobial, antilarvicidal, analgesic, antipasmodic, hypotensive, nematicidal, cholagenic and anticancer properties (Jabeen *et al.*, 2016; Kashif *et al.*, 2015). This compound was also used as a capping and reducing agent to synhesize in one pot gold nanoparticles capped with patuletin. This formulation reduced levels of toxicity of reagents (Ateeq *et al.*, 2015). *Tagete patula L.* is well described in the literature and some studies explore the biocide potential of its essential oils. The composition of major EOs identified in *T. patula L.* is presented in Table 1; some examples are (*Z*)-ocimene **30**, (*Z*)-tagetenone **31** and caryophyllene **32** (Prakash *et al.*, 2012). EOs show some biological activities such as mosquito repellent, fungicidal, insecticidal, acaricidal and larvicidal (Faizi *et al.*, 2011; Gillij *et al.*, 2008; Politi *et al.*, 2013). Compounds **28**-**32** are presented in the Figure 8.

Table 1. Composition in % of the major EOs present in *T. patula L.* (Prakash *et al.*, 2012)

Essential oils	m/m (%)
Limonene	2.1
(*Z*)-ocimene	19.9
(*E*)-tagetone	1.4
(*Z*)-tagetone	1.8
Isoborneol	3.5
(*Z*)-tagetanone	12.4
(*E*)-tagetanone	10.4
Pipertitenone oxide	5.8
Cayophyllene	15.1
(*E*,*E*)-farnesene	2.5

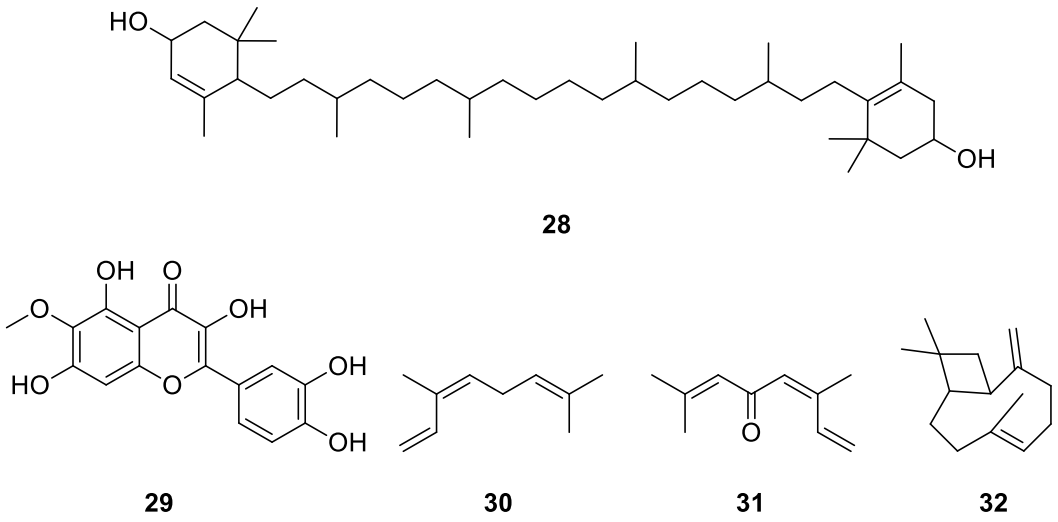


Figure 8. Structures of the main constituents of French marigold: lutein **28**, patulein **29**, (*Z*)-ocimene **30**, (*Z*)-tagetenone **31**, caryophyllene **32**.

1.3.3 *Cotoneaster horizontalis Decne*

Cotoneaster horizontalis Decne (Rock cotoneaster or Rock-Spray) belongs to the family *Rosaceae* and is native from China. Is now spread over Japan, Australian, New Zealand, most of Europe, USA and Canada ("*Cotoneaster horizontalis* (wall-spray)", 2018). Rock-Spray is semi evergreen, low shrub, its branches are spreading horizontally near the ground, the leaves are round to broadly elliptic, 0.25 to 0.5 inch long, dark glossy green, its flowers are pinkish to white colour and the fruits are bright red (Figure 9) (Slabaugh *et al.*, 1990).

This species grows in rocky places and in urban areas, in cracks and in stone walls, hence its name ("Cotoneaster horizontalis (wall-spray)", 2018). Rock cotoneaster are used traditionally as cooling, laxative, astringent, expectorant agents, for the treatment of eye diseases, abdominal pain, piles, bronchitis, itch, thirst, leukoderma, fevers and wounds (Khan *et al.*, 2014).

Figure 9. Morphology of *Cotoneaster horizontalis Decne.*

C. horizontalis Decne extracts are used as expectorant, anticancer, antioxidant, hepatoprotective, antispasmodic, antiviral and diuretic. Phytochemical analysis revealed the presence of some flavonoids, phenolics acids, cyanogen glycosides and tannins. In this species, compounds like α-tocopherol **33** (which presents antioxidant activity) and amygdalin **34** (which presents anticancer activity) have been identified *(Sokkar et al.*, 2013). Mohamed *et al.* (2012) performed HPLC analysis and identified 8 polyphenolic contents (*e.g.* pyrogallol **35**) and 3 flavonoids (*e.g.* luteolin **36**). GLC analyses were also performed and unsaponifiable matters (*e.g. n*-triacontane) and fatty acids (*e.g.* oleic acid **37**) were identified. In this study, the mucilage content of the aerial parts of rock-spray exhibited hypoglycemic and anti-dyslipidemic activities, which might be helpful in treating diabetic conditions associated with atherosclerosis or hyperlipidemia. Rock cotoneaster is also constituted by aromatic esters horizontoate, which can have inhibitory effects on acetylcholinesterase (AChE) and butylcholinesterase (BChE) as well as can have an important biological activity in Alzheimer's disease (Khan *et al.*, 2014). Rock-spray extracts were also used as a phyto-reducer and capping agent for the synthesis of gold nanoparticles. These nanoparticles exhibited antibacterial activity against *P. aeruginosa* (Khan, Runguo, Tahir, Jichuan, & Zhang, 2017). All the compounds referred above are shown in the Figure 10.

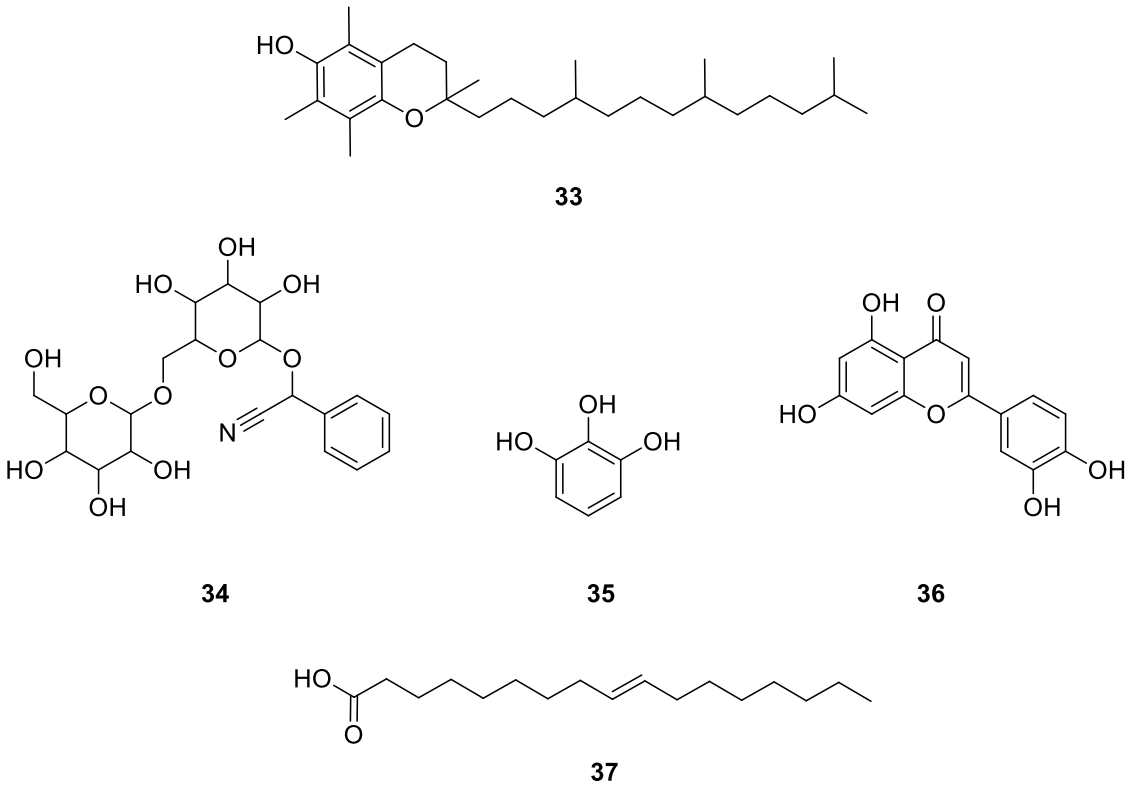


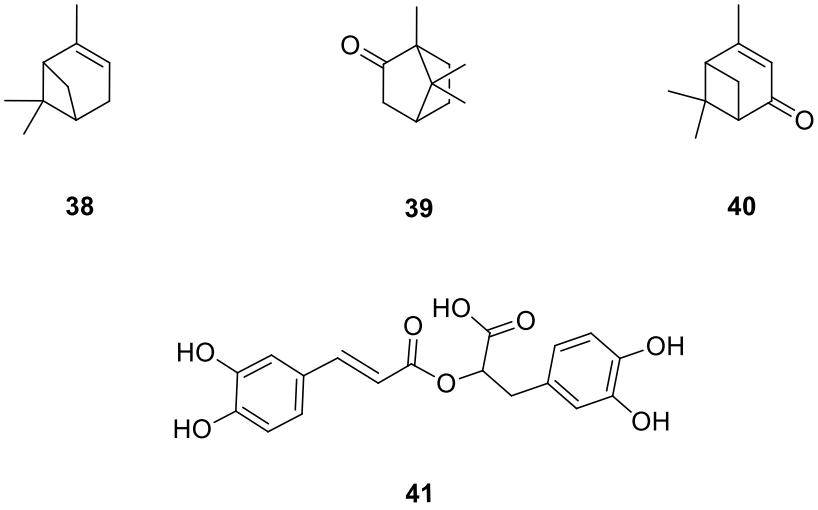
Figure 10. Structures of the main constituents of Rock cotoneaster: α-tocopherol **33**, amygdalin **34**, pyrogallol **35**, luteolin **36**, oleic acid **37**.

1.3.4 *Rosmarinus officinalis L.*

Rosmarinus officinalis L. (Rosemary), belongs to the family *Lamiaceae*, is native from Mediterranean region and now is widely distributed all over the world mainly due to its culinary, medicinal, and commercial uses including the fragrance and food industries. Rosemary is a perennial shrub and usually grows to about 1 metre in height, though some plants can reach up 2 metres. The linear leaves are about 1 cm long and somewhat resemble small curved pine needles. They are dark green and shiny above, with a white underside and curled leaf margins. The small bluish flowers are borne in axillary clusters and are attractive to bees (Figure 11) ("Rosemary", 2018).

Figure 11. Morphology of *Rosmarinus officinalis L.*

Rosemary oil has an extensive use in traditional medicines, since it contains tonic, dietary, antiseptic, antidiarrheal and antirheumatic stimulant properties. In cosmetics, creams with glycolic extracts of the flowering plant are useful in the treatment of stretch marks and also in seborrheic dermatitis. Hydroalcoholic leaf lotions are used to combat dandruff, baldness and to darken hair. The essential oil is also used in perfumery (Özcan *et al.*, 2008). Main constituents of rosemary essential oil are *α*-pinene **38** (up to 30%), *β*-pinene (2 to 6%), camphor **39** (15 to 25%), and in smaller amounts myrcene, *p*-cymene, borneol, verbenone **40**, with the composition varying according to the time of year and the geographic region; flavonoids (luteolin **36**, apigenin, diosmetine and others); polyphenolic acids and caffeic acid derivatives (rosmarinic acid **41**); tannins; bitter constituents of diterpene lactones (carnosol, rosmanol, etc.); triterpenic acids (ursolic and betulinic acid) and triperpenic alcohols (α-amirin, β-amirin and betulin). Rosemary diterpenes have shown in recent years to inhibit neuronal cell death induced by a variety of agents both in *vitro* and in *vivo*. EOs are also responsible for various pharmacological effects as antioxidant, antimicrobial and anticancer activities (Habtemariam, 2016; Moore, *et al.*, 2016). Main constituents of rosemary are presented in Figure 12.



38 **39** **40**

41

Figure 12. Structures of the main constituents of rosemary: α-pinene **38**, camphor **39**, verbenone **40** and rosmarinic acid **41**.

1.3.5 *Aloe barbadensis Miller*

Aloe barbadensis Miller is widely used nowadays in medicine and cosmetics; in antiquity was used for many other purposes, from the treatment of wounds and contact dermatitis to the reduction of hair loss and the elimination of hemorrhoids (Scala *et al.*, 2013). This plant has triangular leaves with toothed edges, and it is possible to distinguish three different layers (Figure 13): the "bark" is a layer of cells with protective function and is involved in the synthesis of carbohydrates and proteins; the clear gel within the plant consists of 99% water (pH 4.5), the remaining 1% being reserved for the polysaccharides (*e.g.* pectin) and the yellowish sap is a latex medium consisting essentially of anthraquinones and glycosides (Raksha, 2014).

Figure 13. Morphology of *Aloe barbadensis Miller*.

The chemical composition of this plant consists of several compounds having specific effects: glucomananas **42** and gibberellin **43** (healing properties); metallothionein (antioxidant); acemannan (effects on the immune system – anticancer); anthraquinones: aloin **44** and emodin **45** (laxative, antiviral, antitumor, antiseptic effects) (Raksha, 2014), (Figure 14). Anthraquinones are a class of compounds which in the case of the *Aloe vera* plant are found in gel and possess a wide variety of pharmacological activities, such as anti-inflammatory, analgesic, antipyretic, antimicrobial, antitumor, wound treatment, among others. As far as anticancer activity is concerned, the highlight is on aloin **44**, since it has the ability to prevent tumor growth, blocking the formation of new blood vessels. On the other hand, emodin **45** is notable for being able to reduce the production of cytokines in endothelial cells and T lymphocytes and to have antiproliferative effects in several cancer cell lines, through apoptosis (Alves *et al.*, 2004; Radha *et al.*, 2015).

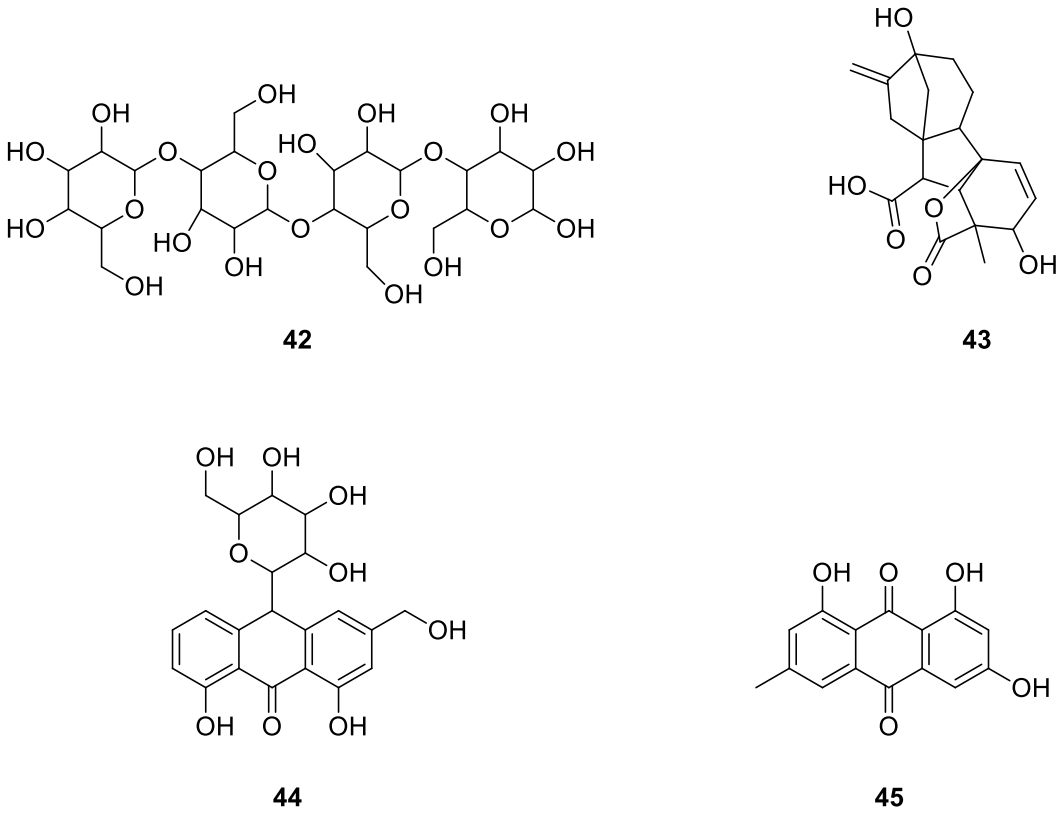


Figure 14. Structures of the main constituents of *Aloe barbadensis Miller*: glucomananas **42**, gibberellin **43**, aloin **44** and emodin **45**.

However, in parallel with the high range of beneficial effects, this plant also has side effects. In contact with the skin it can cause redness, burns, burning sensation or allergic reactions (mainly due to anthraquinones). When ingested orally, it can cause cramps, diarrhea, hepatitis, red urine, an increased risk of colorectal cancer, or an electrolyte imbalance, which in turn will cause a laxative effect.

1.4 Methods of extraction of phytochemicals

Extraction of phytochemicals is the separation of the bioactive compounds from the plant tissues using a specific solvent. There are several extraction techniques, which have impact on the amount and composition of the extract. The extract composition will also depend on the extraction time, the temperature and the nature of the solvent (Ncube *et al.*, 2008). There are various techniques using organic or aqueous solvents; usually solvent extraction is based on either liquid-liquid or solid-liquid extraction.

1.4.1 Hydrodistillation

Hydrodistillation is the simplest and most traditional method. In this method, the vegetable material is placed in an alembic with boiling water. There is a direct contact between water and plant material. This technique can be applied to obtain volatile oils that are less dense than water. An adaptation of the technique uses a Clevenger apparatus, which allows the volatile oils to be accumulated on the top of the water phase, and the amount obtained can be measured directly, without need of additional decantation. During heating and at atmospheric pressure, the water and the EOs form a heterogeneous mixture that reaches the boiling point at a temperature below 100ºC. Then, the heterogeneous mixture is distilled and collected in another flask. As the water is immiscible with the EOs, the two liquids can be easily separated by simple decantation (Figure 15) (Samadi *et al.*, 2017).

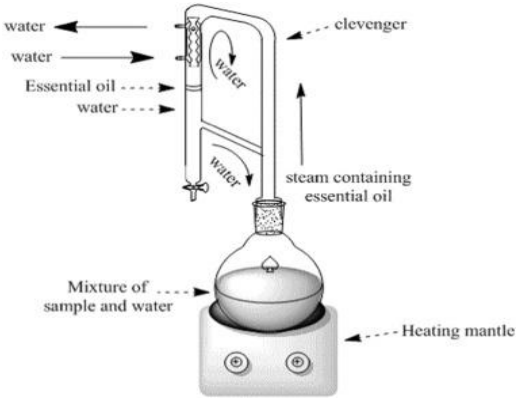


Figure 15. Hydrodistillation using a Clevenger type apparatus (Samadi *et al.*, 2017).

1.4.2 Soxhlet extraction

When the compound has low solubility in a solvent, the best method to be used is Soxhlet extraction. This technique uses a flask with organic solvent and a Soxhlet, which have a cellulose cartridge where the plant material is placed (Figure 16). The solvent is refluxed for the required time and the contents of the cartridge are sometimes replaced with new plant material to obtain a larger amount of extract (Sampath *et al.*, 2017; Cumpson *et al.*, 2013). In this technique the plant material is in permanent contact with fresh solvent, leading usually to good yields of extraction. A disadvantage of this technique is related with the use of organic solvent, which in certain conditions may cause chemical changes.

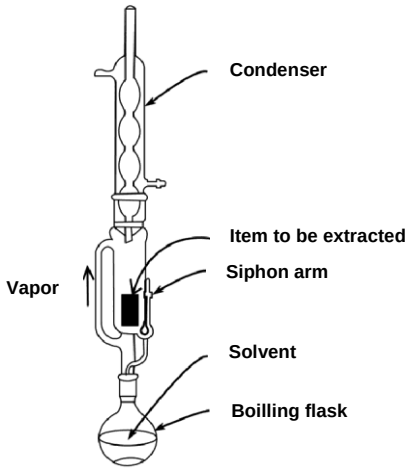


Figure 16. Soxhlet extraction apparatus (Cumpson *et al.*, 2013)

1.4.3 Serial exhaustive extraction

Serial exhaustive extraction is a method involving the successive extraction of compounds by adding a gradient of solvents, in which polarity is increasing. This technique is used to increase the range of compounds to be extracted (Das *et al.*, 2010) and in some cases a selective separation based on the polarity of phytochemicals and solvent is obtained.

1.4.4 Other techniques

There are other modern techniques used to extract phytochemicals from plant material such as: cold pressing, supercritical carbon dioxide extraction, ultrasound assisted extraction and microwave assisted extraction. In cold pressing, oil sacs break and release volatile oils. Thus, the oil is mechanically removed by cold compression producing an aqueous emulsion (Ferhat *et al.*, 2007). In supercritical fluid extraction, carbon dioxide is used to extract essential oils (Moyler, 1993). Ultrasound allows intensification and selective EOs extraction by increasing the permeability of cell walls and accelerating their release from plant material when used in combination with other techniques (hydrodistillation, for example) (Romanik *et al.*, 2007). Microwave assisted extraction is a method that uses microwaves for extraction of essentials oils, and can be combined with other techniques (Sahraoui *et al.*, 2008).

1.5 Identification and characterization techniques

After extraction of phytochemicals, in which crude extracts (complex mixtures) are obtained from natural sources, it is necessary to analyze their composition in order to obtain information on the composition and properties of the extracts. There are many analytical tests and characterization techniques available to obtain structural information, some of the most important are presented below:

1.5.1 Phytochemical screening assay

Phytochemical screening assay is a simple, quick, and inexpensive procedure that identifies the various types of phytochemicals in a mixture. After obtaining the crude extract from plant material, phytochemical screening can be performed with the appropriate tests (all based in Prashant *et al.*, 2011) to get an idea regarding the type of phytochemicals existing in the extract mixture or fraction.

Phytochemical tests for glycosides

The following tests can detect the presence of different types of glycosides. Extracts are hydrolysed with diluted HCl, and then subjected to test for the presence of glycosides. There are many tests to detect different types of glycosides, but the focus is on what is of interest for this study.

To detect the presence of anthraquinone glycosides, there is the Borntrager's Test in which extracts are boiled with dilute sulfuric acid and filtered. To the cold filtrates are added equal volumes of chloroform. After thoroughly shaking, the organic solvent layers were separated and ammonia solution is added. The change of ammonia layer to pink or red color indicates the presence of anthraquinone glycosides.

To detect the presence of saponin glycosides, there are two tests types: Foam Test (0.5 mg of extract is shaken with 2 mL of water. If foam produced persists for ten minutes it indicates the presence of saponins) and Froth Test (extracts are diluted with distilled water to 20mL and are shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins) (Prashant *et al.*, 2011).

Phytochemical tests for steroids and terpenoids

To detect the presence of steroids and triterpenoids, there is Liebermann-Burchard reaction in which chloroform is added to the extracts, mixed, and then acetic anhydride is added followed by concentrated sulfuric acid from the sides of the tubes. Appearance of first red, then blue and finally green color indicates the presence of steroids and triterpenoids.

To detect the presence of diterpenes, there is Copper Acetate Test, in which extracts are dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes (Prashant *et al.*, 2011).

Phytochemical tests for phenolic compounds, tannins and flavonoids

To detect the presence of phenolic compounds and tannins, small quantities of the extracts are treated with the following reagents and the appearance of corresponding endpoints indicate the presence of phenolic compounds and tannins (with 5% ferric chloride solution→deep blue-black color; with 10% lead acetate solution→white precipitate; with 10% potassium dichromate solution→red precipitate).

To detect the presence of flavonoids, there are two tests: Alkaline Reagent Test (extracts are treated with a few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids) and Lead Acetate Test (extracts are treated with a few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids) (Prashant *et al.*, 2011).

1.5.2 Analytical characterization techniques

Most of the analytical methods used in the analysis of the chemical composition of plant extracts are based on chromatographic procedures, which enable the separation and identification of constituents. However, often additional evidence for confirmation is necessary for a complete identification, avoiding deficient characterization. In this context, the use of chromatographic techniques is complemented by other powerful techniques such as NMR, IR and UV-Vis spectroscopies and mass spectrometry.

Different types of chromatographic methods can be applied, from a simple TLC, to GC or LC, which very often are coupled to mass spectrometers. In chromatographic analysis, the compounds to be analyzed are applied on a stationary phase (*e.g.* column chromatography) and eluted by the mobile phase through the column. The analytes are separated on the basis of their specific characterization, such as volatility, polarity, molecular weight, etc. The information obtained in a chromatogram is used either for qualitative or quantitative analysis.

Spectroscopic techniques are very useful, since they can give further structural information (*e.g.* NMR). Finally the coupling of different powerful techniques (mass spectrometry or NMR spectroscopy) to liquid (LC) or gas (GC) chromatography

provides powerful tools for determination of chemical composition of plant extracts. The most common analytical techniques used for characterization of phytochemicals are: liquid chromatography (LC), gas chromatography (GC), thin-layer chromatography (TLC) and fourier-transform infrared spectrometry (FTIR).

The choice of the detection method will depend on the physicochemical properties of the compounds to be analyzed. Spectroscopy is the most used method in the detection of phytochemicals. Ultraviolet-visible (UV-Vis) spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Fluorescence is a spectroscopy method of analysis where the molecules of the extract are excited by irradiation at a certain wavelength and emit radiation of a higher wavelength. Infrared (IR) spectroscopy is used to determine the functional groups present in the sample. Mass Spectrometry (MS) is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds and to elucidate the structure and chemical properties of molecules. Finally, Nuclear Magnetic Resonance (NMR) spectroscopy is a physical measurement of the resonances of magnetic nuclear, such as ^{1}H or ^{13}C in a strong magnetic field; each compound has a specific spectrum. ^{1}H-NMR gives information about how many types of hydrogen atoms are present in the compound and how the hydrogen atoms are connected. ^{13}C-NMR identifies how many types of carbon atoms present in the compound (Banu *et al.*, 2015; Sotelo *et al.*, 2008).

1.6 Biological activity

Plant extracts have biological properties that can be useful to in the pharmaceutical industry. The main biological properties of these compounds are cytotoxic, antimicrobial, antioxidant and anticancer activity (Lubbe *et al.*, 2011).

1.6.1 Cytotoxicity activity

Depending on the concentration, the extracts may demonstrate cytotoxic effects on cells. This property may be used against many pathogens in the pharmaceutical and

veterinary fields as well as in the preservation of agricultural and marine products (Bakkali *et al.*, 2008).

1.6.2 Anticancer activity

In an indirect way, extracts can exert anticancer effects because there is a direct relationship between the production of reactive oxygen species (ROS) and the oxidative and inflammatory stress that can lead to cancer (Klaunig et al., 2011). These compounds have anticancer activity through the induction of apoptosis or cell cycle arrest in cancer cells, the activation of the detoxification and DNA repair systems, and the inhibition of metastasis and angiogenesis.

1.7 Encapsulation of plant extracts

The main problem in application of plant extracts in food, pharmaceutical and cosmetic industries is their extremely sensitivity to oxygen, light, moisture and heat. One strategy to solve this problem is the incorporation of the bioactive compounds in encapsulation systems. Encapsulation is a process of coating or entrapping, by droplets, of active substances (core material) into a material or system (wall material) to give small capsules which can preserve these compounds (Fang *et al.*, 2010). The encapsulation of bioactive compounds also modulate their release, increase the physical stability, protect from oxidation reactions with the environment, decrease volatility, enhance bioactivity, reduce toxicity, protect plant extracts from enzymatic degradation and ensure to them an optimal pharmacokinetic profile *(Bilia et al.*, 2014). There are several different systems for the encapsulation of bioactive compounds. The selection of these systems can depend on size, shape or nature, according to the intended usage of the final formulation. Lipid-based nanostructures will be the encapsulation systems to be explored in this project.

1.7.1 Lipid-based nanosystems

There are three types of lipid-based nanostructures: lipid nanoparticles, liposomes and nanoemulsions. In this project, liposomes will be used as encapsulation systems for the isolated plant extracts.

Liposomes (LPs) are spherical structures that are formed by lipid bilayers enveloping an aqueous volume inside. The lipid bilayer is formed because the character of phospholipid molecules, which is amphiphilic. So, lipophilic chains are facing each other and the hydrophilic heads are in contact with the aqueous medium (Sherry *et al.*, 2013). Hydrophilic substances are encapsulated in the inner aqueous compartments, while lipophilic compounds are mainly entrapped within lipid bilayers and amphiphilic molecules are located between these two regions (Figure 17) (Li *et al.*, 2014). The main advantages of LPs are the possibility of large-scale production, biocompatibility, biodegradability and targetability. However, LPs can exhibit some disadvantages, such as rapid release of the entrapped compounds, high costs (process and materials) and poor loading efficiency (Laouini *et al.*, 2012). There are several techniques for the preparation of liposomes, such as thin lipid film hydration, freeze–thaw, freeze drying and ethanolic injection. In this project, ethanolic injection method will be the preparation technique to be explored, considering the reported efficiency in encapsulating hydrophobic molecules (Jaafar-Maalej *et al.*, 2010) and the characteristics of plant extracts.

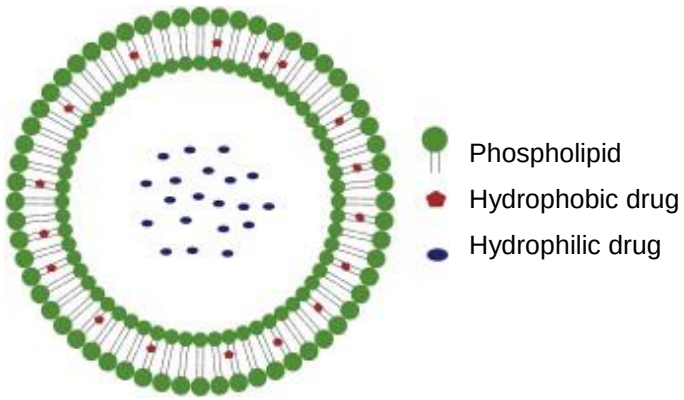


Figure 17. Schematic representation of a liposome (LP) loaded with hydrophilic and hydrophobic drugs.

The ethanolic injection method consists in obtaining small liposomes with a narrow size distribution by simply injecting an ethanolic lipid solution in an aqueous media. The lipid phase is usually injected by means of a syringe pump in a defined volume of ultrapure water (or buffer) under strong vortexing. Then, the spontaneous liposomes are formed. Finally, the ethanol and a part of water can be removed by rotary evaporation under reduced pressure. The main advantage of this technique is that it is easy to scale up (Jaafar-Maalej *et al.*, 2010). The lecithin chosen for the formation of the liposomes, natural and economical, has already demonstrated a good potential in the encapsulation of textile dyestuffs for dyeing (Baptista *et al.*, 2004; De Sousa *et al.*, 2011).

1.7.2 Determination of encapsulation efficiencies

After the encapsulation system is chosen, there are some characteristics that must be taken into account such as: loading capacity, encapsulation efficiency, percentage yield, particle size, polydispersity index, zeta-potential, morphology and stability. The encapsulation efficiency is a measure of the ability of the delivery system to retain the encapsulated bioactive compound. This parameter can be quantified by UV-Vis absorption or fluorescence spectroscopy (if the extract includes fluorescent compounds) (Rodríguez *et al.*, 2016).

One of the primary purposes of the encapsulation of bioactive compounds is to provide their controlled release, and so, release mechanisms of the bioactive compounds by the nanosystems are also important. The efficiency of the controlled release depends on the characteristics of the vehicle, the physicochemical properties of the bioactive compound and several factors related to the interaction of the vehicle system – bioactive compound (Siegel *et al.*, 2012).

1.8 Objective

This study aims to extract and characterize phytochemicals of some plant species (selected based on the criteria of being less studied and distributed in north region of Portugal). The chosen species were *Phytolacca americana L.*, *Tagete patula L.*, *Cotoneaster horizontalis Decne*, *Rosmarinus officinalis L.* and *Aloe barbadensis Miller.* The chemical composition of extracts will be studied using different analytical techniques, including NMR, IR, UV-Vis absorption spectroscopy, HPLC chromatography and mass spectrometry. Biological activity, namely the anticancer potential of these extracts, will be evaluated and their encapsulation using lipid nanosystems will be carried out, in order to improve the stability and mechanism release of these phytochemicals.

Chapter 2: Experimental

2.1 Plant materials

Plant materials (leaves and stems) of *Phytolacca americana L.* and *Tagete patula L.* were collected in S.Paio – Melgaço - Portugal, in September 2017. Leaves of *Rosmarinus officinalis L.* were collected in Tenões- Braga- Portugal, in September 2017. Plant materials of *Cotoneaster horizontalis Decne* were collected in Leça da Palmeira- Porto- Portugal, in April 2018 and leaves of *Aloe barbadensis miller* were always collected on the same day or the day before use in Braga, between May and June, 2018.

2.2 Chemical reagents and characterization methods

All solvents used for extraction, isolation and characterization were provided by Fisher Chemical and Panreac. The silica gel used for thin-layer chromatography (TLC) was provided by Macherey-Nagel on 0.20 mm thick silica gel plates and, to reveal, were used visual detection and ultraviolet light ($\lambda_{máx}$ 254 and 365 nm) in CN-6 chamber. Column and dry-flash chromatographies were performed with Acros Organics 60 silica gel (0.035-0.070 mm). Infrared spectra were recorded on a BOMEM MB 104 spectrophotometer. Nuclear magnetic resonance spectra were determined on the Bruker Avance III 400 apparatus at a frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C, using the solvent peak as an internal reference, at 25°C. The chemical shifts are reported in ppm, using as reference the value δ_H Me_4Si = 0 ppm, and the coupling constants (*J*) occur in Hz. The assignment of the ^{1}H and ^{13}C signals was effected by comparison of chemical shifts, multiplicity of peaks and *J* values. Dual-coupled decoupling techniques were used as well as two-dimensional heteronuclear spectroscopic correlation, namely HMQC and HMBC. The deuterated solvents used were chloroform, dimethylsulfoxide and methanol with deuteration degree higher than 99.8%, from euriso-top®. Ultraviolet-visible spectra (200-800 nm) were obtained on a Shimadzu UV/3101PC spectrophotometer. Fluorescence spectra were recorded on a Fluoromax-4 spectrofluorimeter. Some extracts were analyzed by HPLC-DAD (Gilson), using a reversed-phase Hypersil ODS column. Some samples were also sent to University of Santiago de Compostela (Spain) at the CACTUS building in the unit of Mass

Spectrometry and Proteomics and analyzed by GC-APCI-QTOF (timsTOF) and UHPLC-ESI-TOF (timsTOF).

2.3 Processing of plant material

The plant material was separated according to different parts (leaf, stem, fruit and flowers). In the case of flowers, the flower was also separated from the receptacle. Herbal material, which contained sugars and is more humid, such as fruits, has previously been lyophilized. In this case, pokeweed berries and French marigold flowers (yellow, orange and red) were lyophilized for 7 days in the lyophilizer (Virtis.SP.SCIENTIFIC) at a condenser temperature of -40.2 ºC and a vacuum of 248 mT. The less humid vegetable material was dried in the oven (Heracus) at a temperature of 40-45 ºC for 24h.

The dried plant material was grounded with a chopper (Moulinex) and the resulting powder passed through a sieve. Only powder of plant material less than 910 µm was transferred to a new container properly identified and stored under vacuum for further use.

2.4 Extraction, isolation and characterization

2.4.1 *Phytolacca americana L.*

Soxhlet extraction

The powdered berries of pokeweed (20.00 g) were subjected to soxhlet extraction using ethanol (350 mL) for 3 hours (6 cycles) and then the resulting solution was evaporated under reduced pressure. The obtained extract (5.82 g) was suspended in water, petroleum ether was added, and the organic phase was evaporated. The organic extract was obtained as a green oily-solid **E_1** (843.8 mg), which by TLC revealed the presence of two major components. TLC (petroleum ether/diethyl ether, 3/2, v/v) – major stains R_f = 0.52 and 0.93. ^{1}H-NMR $\delta_H(CDCl_3)$: 5.24-5.42 (~5H, m); 4.30 (~1H, dd, J_1 = 12.0 Hz and J_2 = 4.4 Hz); 4.15 (~1H, dd, J_1 = 12.0 Hz and J_2 = 6.0 Hz); 2.77

(~1H, t, *J* = 6.4 Hz); 2.29-2.35 (~3H, m); 1.97-2.08 (~5H, m); 1.56-1.66 (~4H, m); 1.26-1.38 (~27H, m) and 0.87-0.91 (~5H, m) ppm. ^{13}C-NMR δ_C($CDCl_3$): 173.25 (C=O); 172.83 (C=O); 130.22 (C=C-H); 130.01 (C=C-H); 129.98 (C=C-H); 129.71 (C=C-H); 129.68 (C=C-H); 128.06 (C=C-H); 127.89 (C=C-H); 68.89 (CH); 62.10 (CH_2); 34.19 (CH_2); 34.03 (CH_2); 31.91 (CH_2); 31.53 (CH_2); 29.77 (CH_2); 29.68 (CH_2); 29.63 (CH_2); 29.53 (CH_2); 29.48 (CH_2); 29.35 (CH_2); 29.32 (CH_2); 29.28 (CH_2); 29.18 (CH_2); 29.12 (CH_2); 29.05 (CH_2); 27.22 (CH_2); 27.20 (CH_2); 27.18 (CH_2); 25.63 (CH_2); 24.88 (CH_2); 24.84 (CH_2); 22.69 (CH_2); 22.57 (CH_2); 14.12 (CH_3) and 14.07 (CH_3) ppm. IR $\upsilon_{máx}$ (liquid film): 3007.6; 2925.7; 2854.9; 1745.5; 1463.5; 1377.4; 1239.3; 1163.8; 1098.2 and 727.5 cm^{-1}. HPLC-DAD (methanol:acetonitrile 30:70): RT_1 = 0.67 min; RT_2 = 0.86 min; RT_3 = 1.41 min; RT_4 = 1.73 min; RT_5 = 2.18 min; RT_6 = 2.47 min; RT_7 = 3.11 min; RT_8 = 4.19 min and RT_9 = 6.42 min.

Hydrolysis of extract **E_1**

The previously obtained extract **E_1** (583 mg) was refluxed with 2N H_2SO_4-dioxane (0.69 mL-25 mL) on a water bath for 4 hours. The reaction mixture was diluted with water (25 mL) and extracted with DCM. The organic extract was washed with water, dried with anhyd. Na_2SO_4 and solvent was evaporated, affording a brown solid-oily **E_2** (123.8 mg), which TLC analysis indicated the presence of a major component. TLC (petroleum ether/diethyl ether, 3/2, v/v) – major stain R_f = 0.52. ^{1}H-NMR δ_H ($CDCl_3$): 5.3-5.4 (~1H, m); 2.34 (~2H, t, *J* = 7.6 Hz); 1.95-2.10 (~2H, m); 1.60-1.70 (~3H, m); 1.20-1.40 (~20H, m) and 0.75-0.90 (~4H, m) ppm. The ^{1}H-NMR in DMSO showed a further signal at 11.94 ppm (possibly due to carboxylic acid). ^{13}C-NMR δ_C ($CDCl_3$): 179.56 (C=O); 129.75 (C=C-H); 129.56 (C=C-H); 129.27 (C=C-H); 127.61 (C=C-H); 127.45 (C=C-H); 33.61(CH_2); 31.47 (CH_2); 31.45 (CH_2); 31.07 (CH_2); 29.31 (CH_2); 29.25 (CH_2); 29.23 (CH_2); 29.14 (CH_2); 29.07 (CH_2); 28.99 (CH_2); 28.91 (CH_2); 28.89 (CH_2); 28.87 (CH_2); 28.79 (CH_2); 28.69 (CH_2); 28.62 (CH_2); 28.58 (CH_2); 26.76 (CH_2); 26.70 (CH_2); 25.18 (CH_2); 24.22 (CH_2); 24.16 (CH_2); 24.12 (CH_2); 22.23 (CH_2); 22.12 (CH_2); 13.65 (CH_3) and 13.61 (CH_3) ppm. IR $\upsilon_{máx}$ (liquid film): 3006.6; 2926.0; 2855.6; 2673.8; 1710.8; 1461.6; 1411.3; 1377.9; 1283.0; 1242.2; 1201.4; 1149.8; 940.8;

723.3; 604.1 and 507.4 cm^{-1}. HPLC-DAD (methanol:acetonitrile 30:70): RT_1 = 0.74 min; RT_2 = 0.86 min; RT_3 = 2.00 min and RT_4 = 22.8 min.

2.4.2 *Tagete patula L.*

Hydrodistillation

The powdered yellow flowers of French marigold (3.135 g) were subjected to hydrodistillation (H_2O; 100 mL) during 4 hours. Then, ethyl acetate was added to the aqueous solution. The organic phase was dried and solvent was evaporated under reduced pressure, affording a yellow oily-solid **E_3** (47.3 mg). Deuterated chloroform was added to **E_3** and a precipitate formed, as a brown solid (**F_2**; 30.8 mg) in a yellow solution. The yellow solution was evaporated under reduced pressure to give a yellow solid **F_1** (6.4 mg). **F_2-** TLC (DCM/methanol, 9/1, v/v) – majors stains R_f = 0.06, 0.19, 0.36 and 0.58. ^{1}H-NMR δ_H(MeOD): 6.21-7.90 ppm (aromatic zone); 5.02-5.13 and 3.32-4.10 ppm (olefinic zone), 0.85-1.90 ppm (aliphatic zone). **F_1-** TLC (ethyl acetate) – R_f = 0.54. ^{1}H-NMR δ_H($CDCl_3$): 5.07 (1H, s); 4.97 (1H, s); 4.18-4.21 (1H, m); 3.72 (1H, dd, J_1 = 11.2 Hz; J_2 = 3.6 Hz); 3.57 (1H, dd, J_1 = 11.2 Hz; J_2 = 7.2 Hz); 1.76 (3H, s); 1.22-1.38 (8H, m) and 0.87-0.90 (2H, m) ppm. ^{13}C-NMR δ_C($CDCl_3$): 144.06 ($\underline{C}$=CH_2); 112.03 (C=$\underline{C}H_2$); 75.63 (C-OH); 65.18 (CH_2); 31.91 (CH_2); 29.68 (CH_2); 29.34 (CH_2); 22.67 (CH_2); 18.86 (CH_3) and 14.10 (CH_3) ppm. GC-APCI – major peaks: RT_1 = 3.0 min (169.12 m/z); RT_7 = 6.2 min (137.06 m/z); RT_{12} = 7.8 min (109.10 m/z); RT_{14} = 8.4 min (151.07 m/z); RT_{15} = 8.8 min (151.07 m/z); RT_{17} = 10.2 min (281.25 m/z) and RT_{19} = 10.7 min (235.10 m/z). HRMS data confirmed the exact mass for compounds related RT_1 ($C_{10}H_{15}O$; m/z = 151.1115), RT_{12} ($C_{10}H_{15}O$; m/z = 151.1114) and RT_{17} ($C_{18}H_{33}O_2$; m/z = 281.2477).

Soxhlet extraction

The powdered red flowers of French marigold (865 mg) were subjected to soxhlet extraction with dichloromethane (250 mL) during 4 hours. The solution obtained was evaporated under reduced pressure affording the extract as orange oil **E_4** (154 mg). **E_4** was purified on a silica gel column chromatography and successively eluted with

increasing polarity gradients of petroleum ether/dichloromethane. After combining the fractions with the same R_f values, 6 fractions were finally obtained. The main fractions **F_3** and **F_4** were collected as a red oily-solid (63 mg) and a yellow oily-solid (31 mg), respectively.

F_3 - TLC (petroleum ether/DCM, 4/1; v/v) – majors stains R_f = 0.18, 0.45, 0.66 and 0.82. ^{1}H-NMR $\delta_H(CDCl_3)$: 7.10-7.80 ppm (aromatic zone); 5.00-5.40 and 3.90-4.35 ppm (olefinic zone) and 0.70-2.10 ppm (aliphatic zone).

F_4 - TLC (petroleum ether/DCM, 7/3; v/v; iodine stain) – majors stains R_f = 0.23 (yellow stain), 0.26 (green stain), 0.35 (brown stain) and 0.79. ^{1}H-NMR $\delta_H(CDCl_3)$: 7.20 (d) and 7.10 (d) ppm (aromatic zone); 5.42-5.31 (m), 4.12-4.00 (m), 3.68 (s) and 3.50 (s) ppm (olefinic zone) and 2.90-0.80 ppm (aliphatic zone).

2.4.3 *Cotoneaster horizontalis Decne*

Soxhlet extraction

The powdered leaves of Rock cotoneaster (10.25 g) were subjected to soxhlet extraction with ethanol (350 mL) during 2 hours. The solution obtained was evaporated under reduced pressure to gives a dark green oil **E_5** (1.593 g). The obtained extract was suspended in water and petroleum ether was added; the organic phase was collected and the solvent was evaporated. The extract **E_6** (359 mg) was obtained as a green oil. TLC (DCM/Methanol, 9/1, v/v) – major stains R_f = 0.21, 0.31, 0.43, 0.72 and 0.84. ^{1}H-NMR $\delta_H(CDCl_3)$: 8.09 (d, J = 7.2 Hz); 7.63-7.59 (m); 7.47 (t, J = 8 Hz); 5.40-3.82 ppm (olefinic zone); 2.83-0.84 (aliphatic zone) ppm.

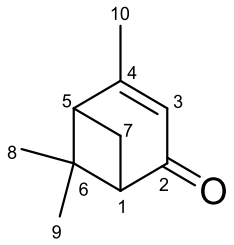
The extract **E_6** was chromatographed on a silica gel column and successively eluted with stepwise gradients of petroleum ether/DCM. The major fraction (**F_5**) (dark green oil) was characterized by TLC and ^{1}H-NMR. TLC (petroleum ether/DCM, 1/1, v/v) – major stains R_f = 0.43, 0.72 and 0.84. ^{1}H-NMR $\delta_H(CDCl_3)$: 7.18 (d, J = 7.6 Hz); 7.14 (d, J = 7.2 Hz); 5.40-3.82 ppm (olefinic zone); 2.83-0.84 (aliphatic zone) ppm.

2.4.4 *Rosmarinus officinalis L.*

Hydrodistillation

The fresh leaves of Rosemary (49.42 g) were subjected to hydrodistillation (H_2O; 250 mL) during 3 hours. To the distilled mixture was added dichloromethane, the organic phase was dried with anhyd. Na_2SO_4 and solvent was evaporated, to give **E_7** as a colorless oil (351 mg), which after purification and characterization was identified as verbenone **40** (60 mg) (Mills, 1996).

The extract **E_7** was chromatographed on a dry-flash column and eluted with ethyl acetate/DCM. **F_6** (verbenone **40**) - TLC (ethyl acetate/ DCM (1/1, v/v) – R_f = 0.52.^{1}H-NMR δ_H($CDCl_3$): 5.73 (sext, J = 1.6 Hz, 1H, H-3), 2.81 (dt, J_1 = 9.6 Hz e J_2 = 5.6 Hz, H-7), 2.65 (td, J_1 = 5.6 Hz e J_2 = 1.6 Hz, H-1), 2.42 (td, J_1 = 5.6 Hz e J_2 = 1.6 Hz, 1H, H-5), 2.08 (d, J = 9.6 Hz, H-7), 2.03-2.01 (m, 10-CH_3), 1.50 (s, 8-CH_3), 1.02 (s, 9-CH_3) ppm.



40

2.4.5 *Aloe barbadensis Miller*

Liquid-liquid extraction

The fresh sap inside leaves of *Aloe Vera* (70 mL) was subjected to a series of liquid-liquid extractions using solvents with increasing polarity (petroleum ether, diethyl ether, ethyl acetate and dichloromethane). The DCM extract was dried with anhyd. Na_2SO_4 and solvent evaporated to give a blue solid **E_8** (10 mg). TLC (DCM/methanol 9/1, v/v) – R_f = 0.5. ^{1}H-NMR δ_H(DMSO) main signals: 9.9 (s,

aldehyde zone); 6.47 (s, aromatic zone); 6.36 (s, aromatic zone); 6.24 (s, aromatic zone), 4.5-5.0 (alkenic zone) and 2.1 (s, aliphatic zone) ppm. UHPLC-ESI-TOF – major peaks: RT_2 = 3.0 min (411.13 m/z); RT_3 = 3.7 min (555.18 m/z); RT_4 = 4.0 min (419.13 m/z); RT_5 = 4.1 min (419.13 m/z); RT_6 = 4.3 min (359.15 m/z); RT_7 = 7.1 min (344.31 m/z) and RT_8 = 7.5 min (325.23 m/z). HRMS data confirmed the exact mass for compounds 4 and 5 shown on HPLC chromatogram, which are related to RT_4 ($C_{21}H_{23}O_9$; m/z = 419.1327) and RT_5 ($C_{21}H_{23}O_9$; m/z = 419.1330).

2.6 Biological assays

2.6.1 Cell cultures

AGS (adenocarcinoma gastric of the stomach) cells were thawed in 45 mL of DMEN(1x)+GlutaMAx™-1 (Dulbecco's Modified Eagle Medium, Gilbco), culture medium enriched with 1% penicillin/streptomycin and 10% FBS (fetal bovine serum) at 37°C. After centrifugation at 210*g* for 5 minutes, the solvent was discarded and the pellet was resuspended in 15 mL of medium at 37°C. To proceed to a 2D culture, a volume of cell suspension (suitable for the desired growth) was added to a 300 mL flask together with 15 mL of culture medium. The conditions described were obtained from da Silva *et al.*, 2017.

2.6.2 Cell viability

MTT

After obtaining about 80% confluency of the container, the medium was discarded and the container washed twice with 5 mL of HBSS (Hank's balanced salt solution) purchased from Gilbco. Subsequently, 2.5 mL of 0.25% Trypsin was placed and the container was brought to 37°C and 5% CO_2 for 3 minutes. Then 5 mL of medium were added and the entire volume of the container was collected and centrifuged at 1300 rpm for 3 minutes at 37°C. The supernatant was discarded and the pellet resuspended in 4 mL of medium.

In an eppendorf, were placed 90 µL of Trypan blue and 10 µL of the cell suspension. A Neubauer chamber was filled with this solution to count the number of cells present in the cell suspension. After 24 hours successive dilutions of the test compound were prepared. The medium from each well was aspirated and 100 µL of each dilution of the extract was added in triplicate.

After removal of the test compound, the MTT substrate was prepared with medium and added to the cultured cells of each well in a ratio of 1:10. The incubation was carried out for 1 hour and one quarter at 37°C and, after that, the MTT solution was removed. The formazan formed was dissolved in 200 µL of a mixture of DMSO: isopropanol (3:1). Absorbance was then measured at 570nm using a Thermo Scientific ™ MultiskanTMGO microplate reader (Zou *et al.*, 2013).

LDH

After 24 hours of incubation of the test compound, as previously described by da Silva *et al.*, 2017, 20 µL of the solution present in each well was withdrawn into a new 96 well plate. 25 µL of a solution of pyruvate and 230 µL of a solution of NADH, both at the concentration of 3mg/20mL, were added. These two solutions were prepared in phosphate buffer at pH 7.4 and 1% Triton X-100 was used as the positive control. The integrity of cellular membrane was measured by the absorbance recorded at 340nm in a Thermo Scientific ™ Multiskan ™ GO microplate reader.

2.7 Encapsulation assays

For the nanoencapsulation assays, the extracts $\mathbf{E_1}$, $\mathbf{E_2}$ and fraction $\mathbf{F_4}$ were used. Dilutions were performed at concentrations of 0.1×10^{-5} – 4.5×10^{-5} M for the calibration curves and determination of the encapsulation efficiency. The quantification was performed through UV-Vis absorption or fluorescence spectroscopy. Liposomes were prepared by the ethanolic injection method (Kremer *et al.*, 1977), using a commercial lipid mixture, soybean lecithin (Sternchemie), containing (% mol/mol) 22% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylinositol and

10% of phosphatidic acid as the major components, at a concentration of 1×10^{-3} M. Ethanolic solutions of extracts/compounds of 2×10^{-5} M concentration were added to the lipid mixture and then pooled dropwise under vortexing in a buffer solution of Tris-HCl pH = 7.3. The formulations were placed in Amicon™ filter tubes and centrifuged for 10 min. at 3000 rpm. The non-encapsulated fraction was evaporated, and 2 mL of ethanol were added. The absorbance of each solution was measured and the concentration of compound that was not encapsulated was estimated through a calibration curve previously obtained using the Lambert-Beer law (equation 1),

$$A = \varepsilon \times l \times c \qquad (1)$$

where *A* is the absorbance, ε is the molar absorption coefficient, *l* is the optical path (1 cm, in this case) and *c* is the concentration of the sample. The encapsulation efficiency, *EE(%)*, was obtained through equation 2,

$$EE(\%) = \frac{Total\ amount - Amount\ of\ nonencapsulated\ extract}{Total\ amount} \times 100 \qquad (2)$$

2.8 Statistical analysis

Cytotoxicity activity of extracts/compounds was statistically analyzed. The viability percentages of the different concentrations of the extracts/compounds were determined and it was verified if they were statistically significant using a control as reference. For this purpose, one-way ANOVA (non-parametric) and Dunnett test (which compares all columns of different concentrations of extracts/compounds with the control column) was used in GraphPad Prism 5 software.

Chapter 3: Results and discussion

This work is part of a recently funded FCT project (Project POCI-01-0145-FEDER-030154) and consists in the isolation and characterization of the chemical composition of some plant extracts, followed by evaluation of their biological activity. Some of the most promising extracts were encapsulated for future biological evaluation. The plant species were selected based on the criteria of being less studied, available in the north region of Portugal and described in the literature. The plant material used was either fresh when possible or previously dried according to standard procedures. The extracts were obtained using either soxhlet extraction or hydrodistillation and were chemically characterized by NMR, UV and IR spectroscopies, by mass spectrometry and by HPLC and GC chromatography. The encapsulation techniques and the biological activity evaluation tests are also discussed in this work.

3.1 Extracts and characterization

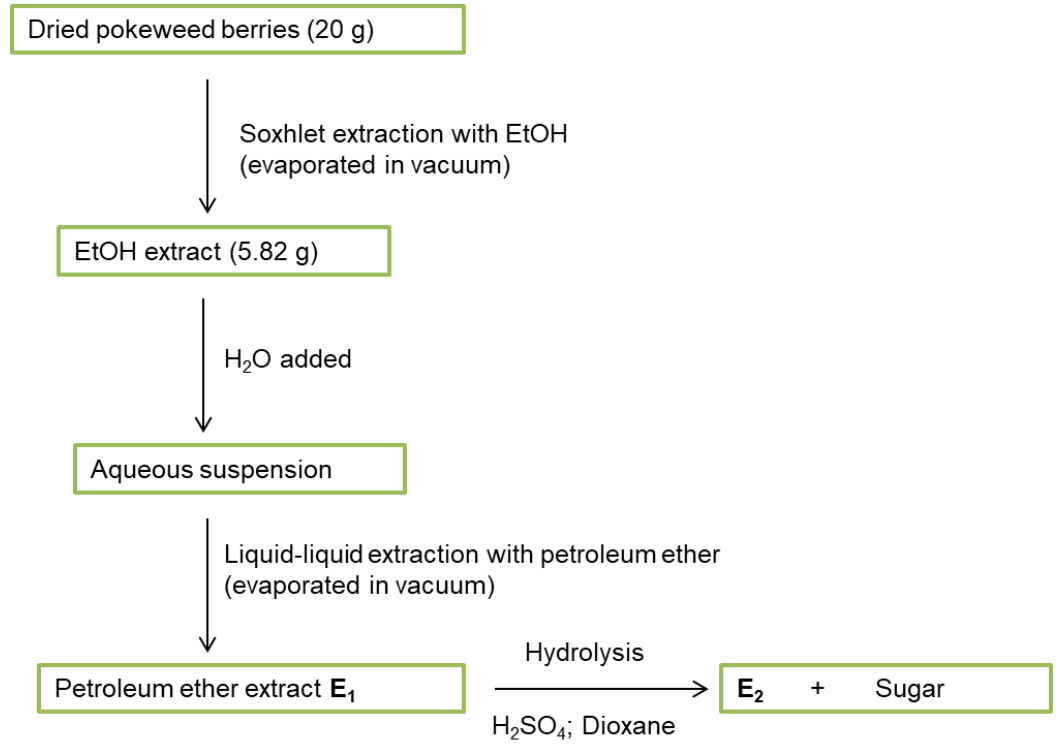
The selection of species was made based on the biological importance, the lack of information in the literature and the fact that the species are available in the north region of Portugal. The extractions and the characterization of the extracts/compounds were compared when possible with the information that exists in the literature on these species.

3.1.1 *Phytolacca americana L.*

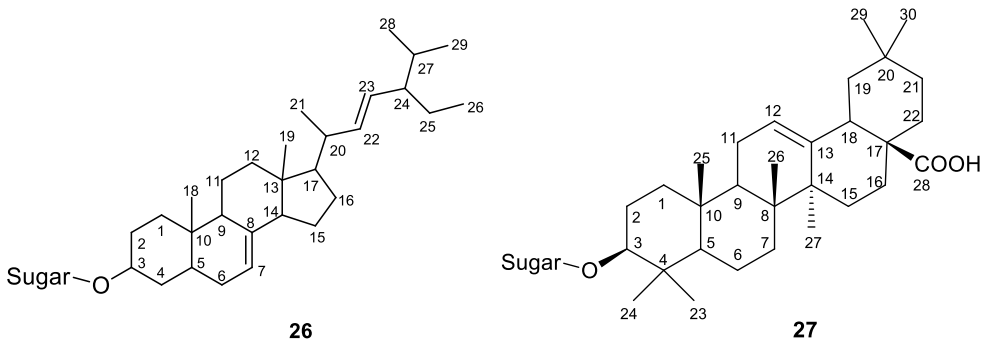
Pokeweed berries were lyophilized and grounded and the powder was subjected to soxhlet extraction using ethanol. The obtained extract was treated according to the procedure indicated in Scheme 1, affording a green oily-solid **E_1** (4%; m/m), which by TLC (petroleum ether/diethyl ether, 3/2, v/v) revealed the presence of two major compounds (R_f = 0.52 and 0.93).

Purification of the extract was attempted by column, dry-flash column and PLC chromatographies, but separation of the two major components was not successful. The extract obtained **E_1** was characterized by HPLC-DAD, UV-Vis, fluorescence, NMR and IR spectroscopies. The HPLC-DAD chromatogram shows the presence of a mixture of

various compounds, in which two are major ones with RT_1 = 0.67 min and RT_2 = 0.86 min. The UV spectra of these two major compounds show a maximum of absorption at 204 nm, which is compatible with a saponin type compound (Figure 19 A, B and C) (Oliveira *et al.*, 2010). The fluorescence spectrum of **E_1** (figure 20) evidences the presence of chlorophyll, with its characteristic emission band with maximum at 690 nm (Pedrós *et al.*, 2008). The main features of ^{1}H-NMR are the presence of a multiplet at δ5.24-5.52 ppm, probably due to a olefinic proton, a set of two double of doublets at δ4.15 ppm (J_1 = 12.0 Hz; J_2 = 6.0 Hz) and δ4.30 ppm (J_1 = 12.0 Hz; J_2 = 4.4 Hz) compatible with the presence of a sugar unit(s), a multiplet at δ1.26-1.38 ppm, integrating for ~26 protons corresponding to CH_2 groups and a multiplet at δ0.87-0.91 ppm, probably due to alkyl groups. The main features of ^{13}C-NMR are the presence of two quaternary carbons at δ173.3 and δ172.8 ppm, probably due to ester carbonyl groups, four peaks at δ127.9-130.2 ppm, which probably correspond to alkenic carbons (C=C-H), a CH carbon at δ68.9 ppm, 22 carbons at δ22.7-62.1 ppm, which correspond to CH_2 groups (confirmed by dept135 in ^{13}C-NMR) and two carbons at δ14.1 and δ14.0 ppm, probably due to alkyl groups. IR spectrum of **E_1** shows bands at 2854.9, 2925.7 and 3007.6 cm^{-1} possibly corresponding to CH groups and a band at 1745.5 cm^{-1} compatible with C=O group. LRMS (ESI NEG) shows the presence a various fragments with m/z difference between them compatible with the presence of sugar units. A sample has been submitted for HRMS analysis, which will give further information about the structure of the right compound. Based on these data and in the literature information (Escalante *et al.*, 2002; Strauss *et al.*, 1995; Suga *et al.*, 1978), **E_1** is compatible with the presence of steroid or triterpenoid saponins (Figure 18 - **26** and **27**).



Scheme 1. Schematic representation of extraction and purification procedure of pokeweed berries.



26 **27**

Figure 18. Structures of possible saponins: steroid **26** and triterpenoid **27**.

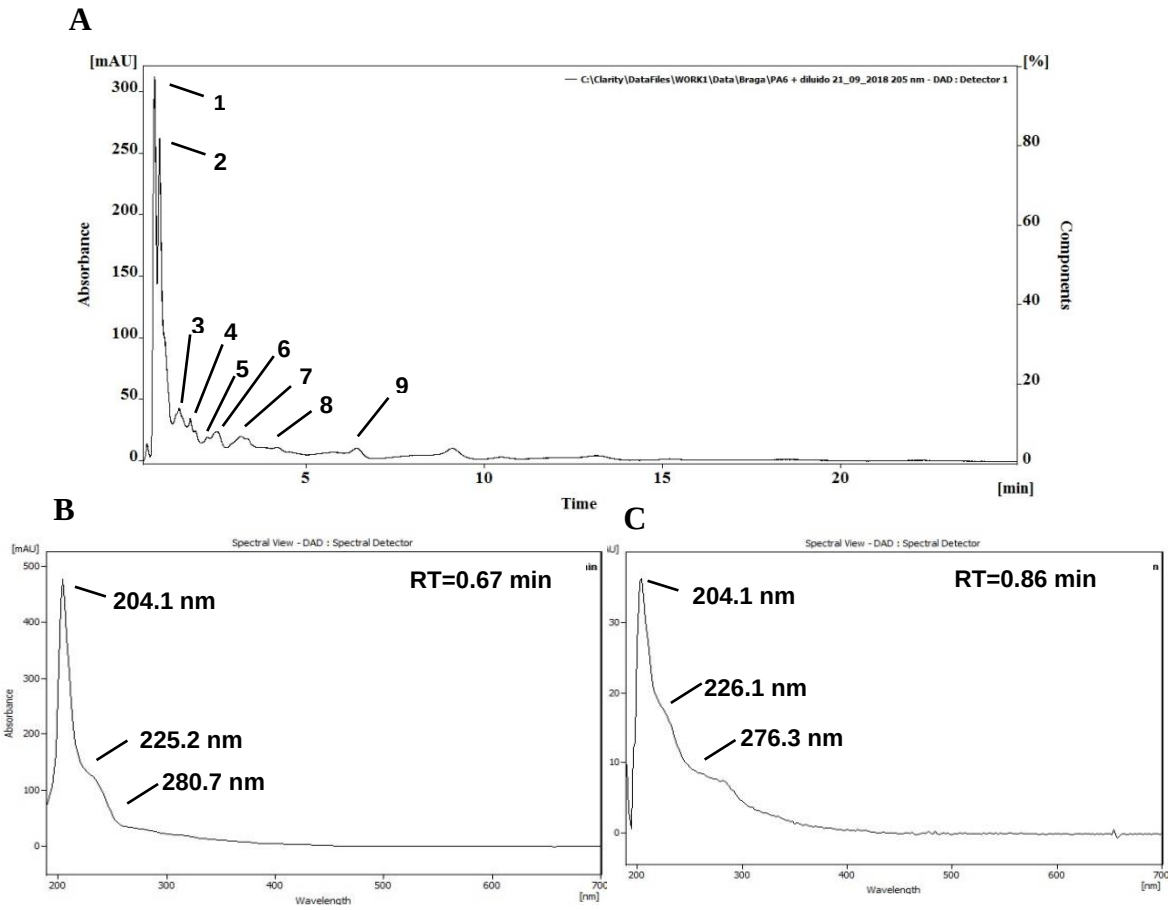


Figure 19. A. Chromatogram (λ = 205 nm) of sample **E_1. B.** UV-Vis absorption of compound 1 in **E_1**. **C.** UV-Vis absorption of compound 2 in **E_1**.

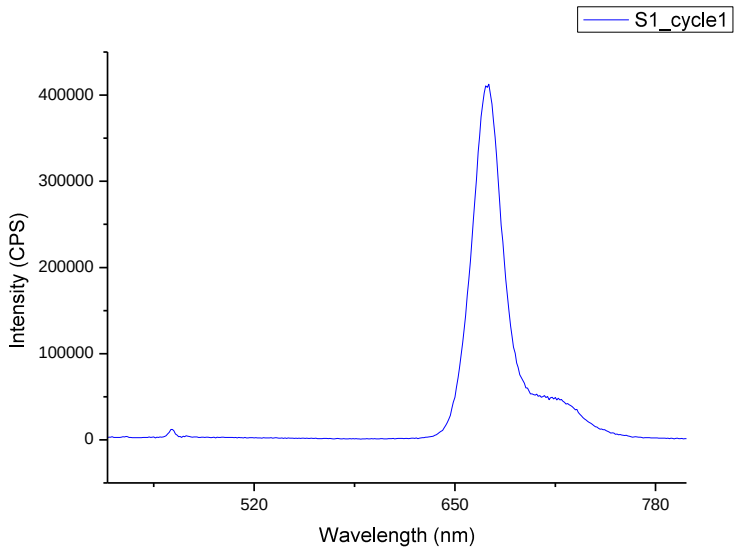


Figure 20. Fluorescence spectrum of **E_1**.

Extract **E_1** was subjected to acidic hydrolysis (Suga *et al.*, 1978) in the presence of H_2SO_4 in dioxane, giving after work-up a residue **E_2** as brown oily-solid (21%; m/m), which by TLC revealed the presence of one major compound (R_f = 0.52; petroleum ether/diethyl ether, 3/2, v/v) (Scheme 1). The reaction mixture **E_2** was also characterized by HPLC-DAD, UV-Vis, NMR and IR spectroscopies. The HPLC-DAD chromatogram shows the presence of 4 compounds including two major ones with RT_1 = 0.74 min and RT_2 = 0.86 min. The UV spectra of these two major compounds show a maximum of absorption at 205 nm, which is compatible with a sapogenin type compound (Figure 21 A, B and C) (Oliveira *et al.*, 2010), indicating that the sugar unit(s) have been cleaved from the corresponding saponine. The fluorescence spectrum of **E_2** exhibits a maximum of emission at 410 nm (Figure 22), however with a low intensity, which can be due to the residual presence of a polyaromatic compound.

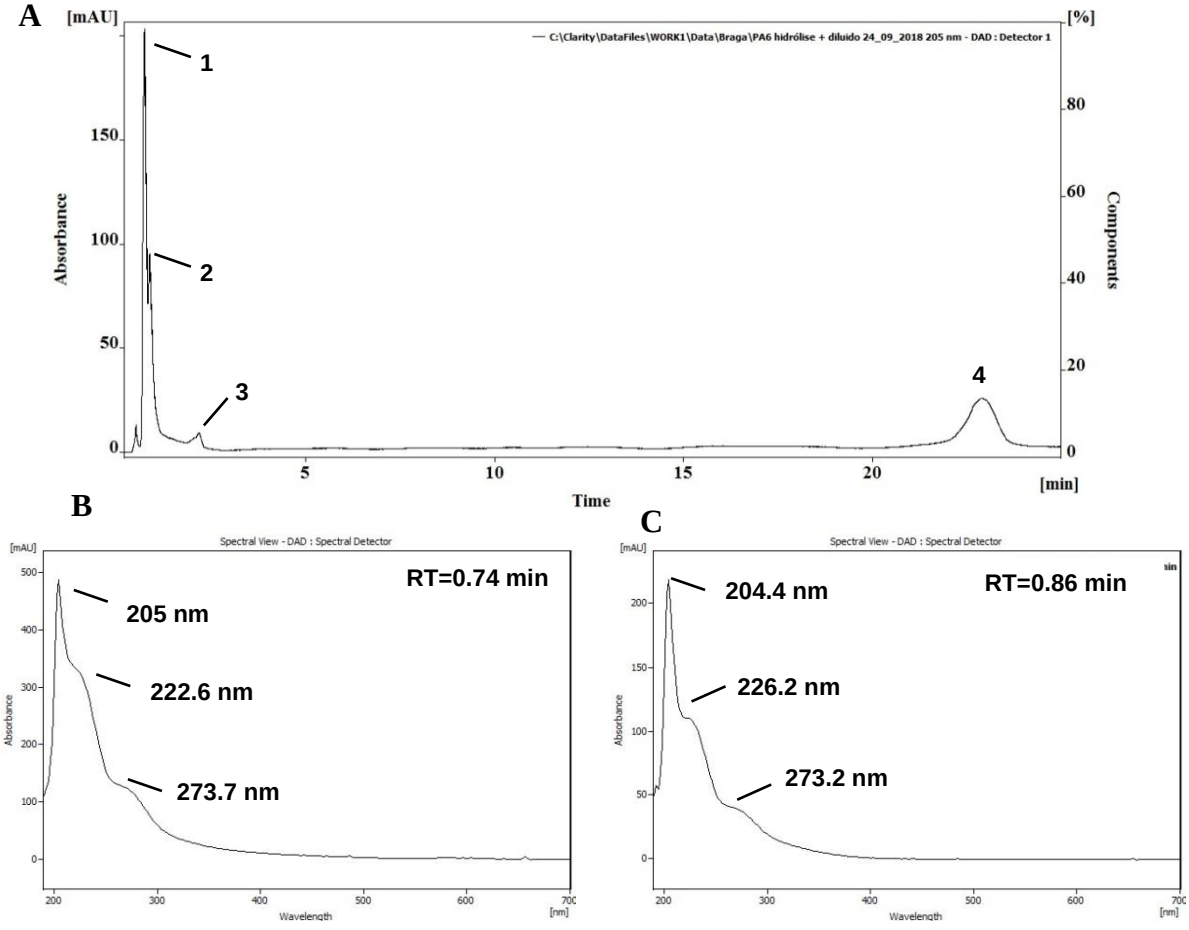


Figure 21. A. Chromatogram (λ = 205 nm) of sample **E_2. B.** UV-Vis absorption of compound 1 in **E_2**. **C.** UV-Vis absorption of compound 2 in **E_2**.

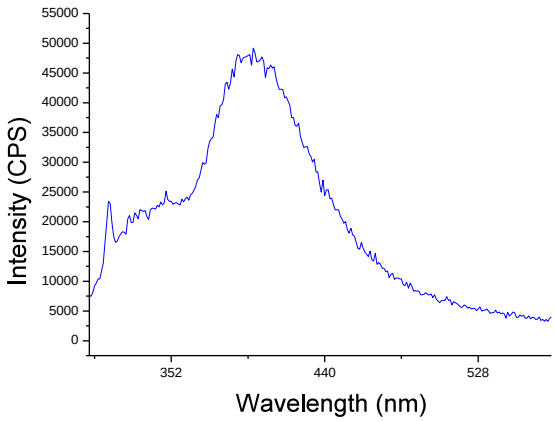


Figure 22. Fluorescence spectrum of $\mathbf{E_2}$.

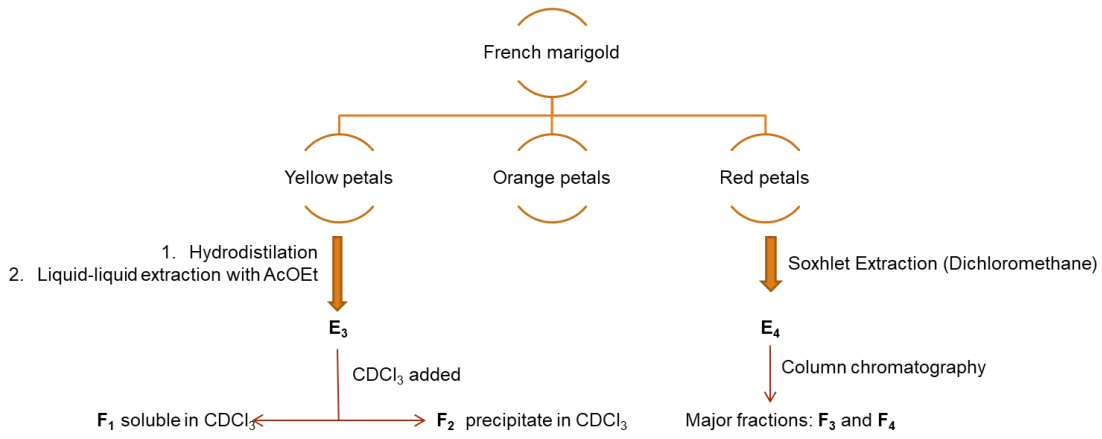
NMR data (^{1}H- and ^{13}C-) of $\mathbf{E_2}$ is very similar to those of $\mathbf{E_1}$, with two major differences in the ^{1}H-NMR, a signal at δ11.9 ppm typical of a COO<u>H</u> group, supporting the cleavage of sugar unit(s), and the disappearance of the two double of doublets at δ4.15 and δ4.30 ppm present in $\mathbf{E_1}$. ^{13}C-NMR main features are a signal at δ179.6 ppm, typical of an acid carbonyl group, and the disappearance of two carbons at δ68.9 (CH) and δ62.1 (CH_2) ppm, which HMQC spectrum shows to be related with the protons of the double of doublets. The IR spectrum of $\mathbf{E_2}$ shows a new broad band over 3000 cm^{-1} also compatible with a CO_2H group. Also based on these data and in the literature information (Escalante *et al.*, 2002; Strauss *et al.*, 1995; Suga *et al.*, 1978) $\mathbf{E_2}$ is compatible with the presence of steroid or triterpenoid sapogenins (Figure 18 - **26** and **27** without a sugar unit(s)). The spectroscopic data obtained for $\mathbf{E_1}$ and $\mathbf{E_2}$ are present in Table 2.

Table 2. Differences between characterization of $\mathbf{E_1}$ and $\mathbf{E_2}$.

Extract	Properties	IR (liquid film) cm^{-1}	Major peaks in ^{1}H-NMR δ($CDCl_3$) (ppm)	Major peaks in ^{13}C-NMR δ($CDCl_3$) (ppm)
E_1	Green oily-solid	3007.6 (C-H) 2925.7 (C-H) 2854.9 (C-H) 1745.5 (C=O)	5.24-5.42 (m, C-H) 4.30 (dd, J_1 = 12 Hz and J_2 = 4.4 Hz, C-H) 4.15 (dd, J_1 = 12 Hz and J_2 = 6.0 Hz, C-H) 1.26-1.38 (m, CH_2) 0.87-0.91 (m, CH_3)	173.2 (C_q) 172.8 (C_q) 127.0-130.0 (C=C-H) 68.9 (CH) 22.5-62.1 (CH_2) 14.1 and 14.7 (CH_3)
E_2	Brown oily-solid	3006.6 (C-H) 2926.0 (C-H) 2855.6 (C-H) 2673.8 1710.8 (C=O)	11.93 (s, COOH) 5.28-5.35 (m, C-H) 1.26-1.38 (m, CH_2) 0.87-0.91 (m, CH_3)	179.5 (C_q) 127.4 – 129.7 (C=C-H) 22.1-33.6 (CH_2) 13.65 and 13.61 (CH_3)

3.1.2 *Tagete patula L.*

Yellow and red flowers of French marigold were lyophilized and grounded and their powder was subjected to hydrodistillation and soxhlet extraction, and purified by solvent precipitation and column chromatography, respectively, as indicated in Scheme 2, affording 4 fractions ($\mathbf{F_1}$, $\mathbf{F_2}$, $\mathbf{F_3}$ and $\mathbf{F_4}$).



Scheme 2. Schematic representation of extraction and purification procedure of French marigold flowers.

F_1 (yellow solid, 14% (m/m)) shows the presence of one compound by TLC (R_f = 0.54; ethyl acetate). ^{1}H-NMR main features are a multiplet at δ0.87-0.90 ppm, corresponding to alkyl groups, a triplet at δ1.28 ppm (J_1 = 10.4 Hz; J_2 = 8.0 Hz), corresponding to 8 protons of methylenic (CH_2) groups, a singlet at δ1.76 ppm, related to an alkyl group, two double of doublets at δ3.57 ppm (J_1 = 11.2 Hz; J_2 = 7.2 Hz) and δ3.72 ppm (J_1 = 11.2 Hz; J_2 = 3.6 Hz), which correspond to 2 protons (CH_2) attached to the same carbon, a multiplet at δ4.18-4.21 ppm, probably due to a proton a CH group and two singlets at δ4.97 and δ5.07 ppm, which corresponding to 2 protons (CH_2) attached to the same carbon. ^{13}C-NMR main features are a quartenary carbon at δ144.1 ppm, possibly due to a $\underline{C}$=CH_2, a carbon at δ112.0 ppm, corresponding to a terminal methylenic carbon, a carbon at δ75.6 ppm (corresponding to a $\underline{C}$-OH group), 5 carbons at δ65.2, δ31.9, δ29.6, δ29.4 and δ22.7 ppm, which correspond to methylenic groups and 2 carbons at δ18.9 and δ14.1 ppm, which are associated to alkyl groups. HMQC spectrum shows that the two double of doublets at δ3.57 and δ3.72 are related to the carbon at δ65.2 ppm and the two singlets at δ4.97 and δ5.07 ppm is related to the carbon at δ112.0 ppm. GC-APCI-QTOF shows a complex mixture (Figure 23), which mass spectrum indicated the presence of compounds with molecular mass ranging from 91 to 281 m/z. APCI-QTOF of compounds 1, 12 and 17 present in the chromatogram of the mixture indicated the presence of monoterpenes and isomers (compound 1, HRMS 151.1115 m/z; compound 12, HRMS 151.1114 m/z and compound 17, HRMS 281.2477 m/z) (Table 3). Based on these data and analysis of some structures, **F_1** is a monoterpene which is constituted by 10 carbons and has a hydroxyl group and a terminal =CH_2.

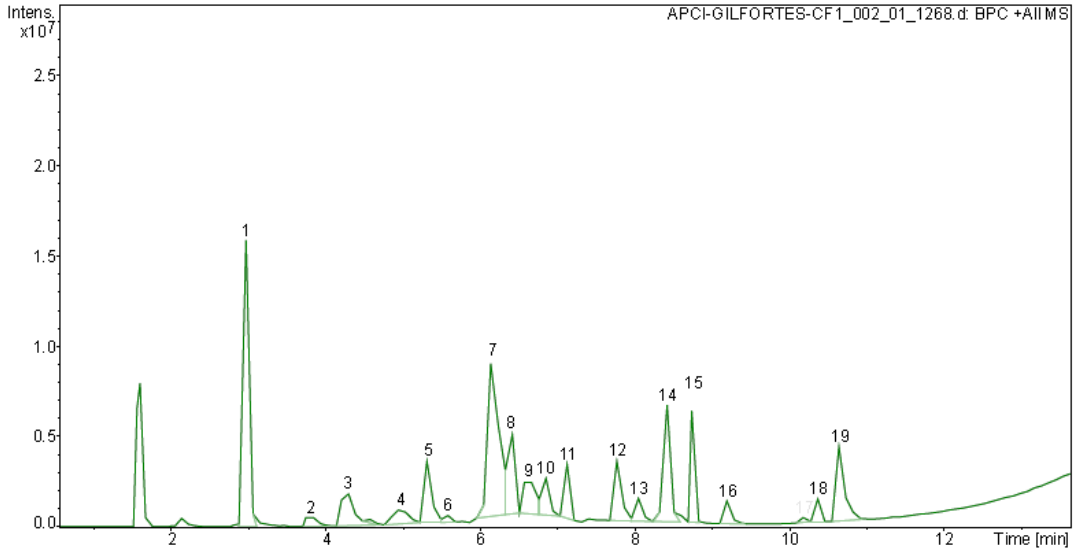


Figure 23. Chromatogram of fraction (**F_1**) of French marigold in GC-APCI-QTOF analysis.

Table 3. Measured and theoretical m/z for each major compounds identified by HRMS in **F_1**.

#	Measured m/z	Ion formula	m/z
1	151.1115	$C_{10}H_{15}O$	151.1117
12	151.1114	$C_{10}H_{15}O$	151.1117
17	281.2477	$C_{18}H_{33}O_2$	281.2475

F_2 (brown solid, 65%, m/m) by TLC (R_f = 0.06, 0.19, 0.36 and 0.58; DCM/methanol, 9/1, v/v) shows the presence of four compounds. ^{1}H-NMR show signals at the aromatic zone (δ6.21-7.90 ppm), at the olefinic zone (δ5.02-5.13 and δ3.32-4.10 ppm) and at the aliphatic zone (δ0.85-1.90 ppm).

F_3 was the major fraction (by mass) after column chromatography (red oily-solid, 18%; m/m) and show the presence of four compounds by TLC (R_f = 0.18, 0.45, 0.66 and 0.82; petroleum ether/DCM, 4/1; v/v). The UV-Vis spectrum shows four bands with maximum absorption at 203, 228, 284 and 360 nm (Figure 24). According to Proestos *et al.* (2006), phenolic acids have a maximum absorption at 280 nm and flavonoids have a maximum absorption at 320 and 370 nm; these data could suggest that **F_3** probably contains phenolic acids and flavonoids type compounds. ^{1}H-NMR show signals at

aromatic zone (δ7.1-7.8 ppm), at olefinic zone (δ5.0-5.4 and δ3.90-4.35 ppm) and at aliphatic zone (δ0.70-2.10 ppm).

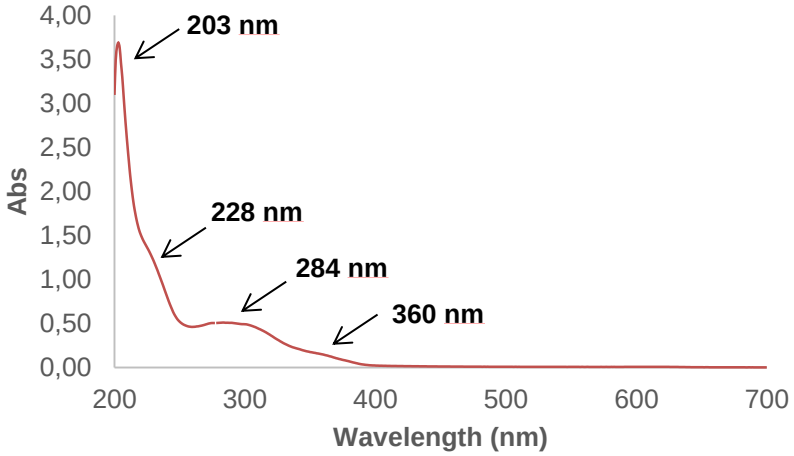


Figure 24. UV-Vis absorption spectrum of **F_3.**

F_4 was the second major fraction (by mass) after column chromatography (yellow oily-solid, 9%; m/m) and show the presence of four compounds by TLC with a iodine detection (R_f = 0.23 (yellow stain), 0.26 (green stain), 0.35 (brown stain) and 0.79; petroleum ether/DCM, 7/3; v/v). The UV-Vis absorption spectrum shows a band with three peaks, with absorption between 400 and 500 nm, which evidences the presence of carotenoids compounds, justifying its yellow colour (Figure 25) (Shindo et al., 2007). ^{1}H-NMR shows signals at aromatic zone (δ7.10 (d) and 7.20 (d) ppm), at olefinic zone (δ5.42-5.31 (m), δ4.12-4.00 (m), δ3.68 (s) and δ3.50 (s) ppm) and at aliphatic zone (δ0.80-2.90 ppm).

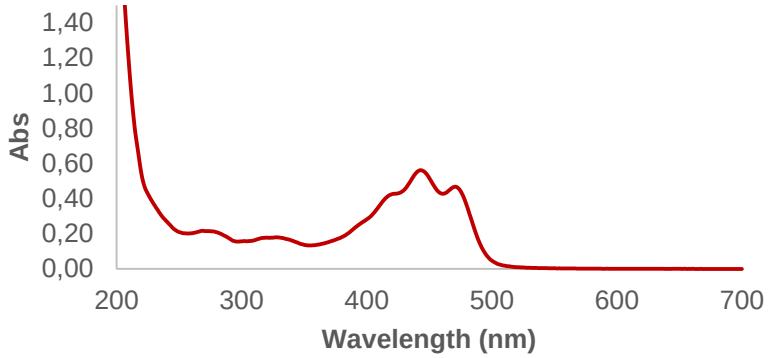
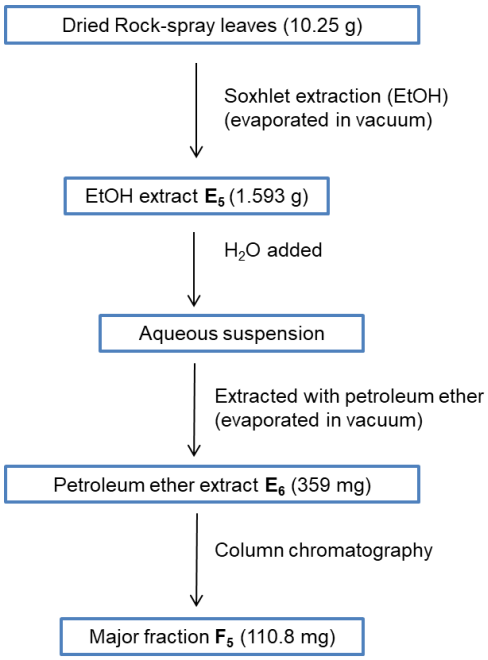


Figure 25. UV-Vis absorption spectrum of **F_4**.

3.1.3 *Cotoneaster horizontalis Decne*

Leaves of Rock cotoneaster were dried and grounded and the powder was subjected to soxhlet extraction using ethanol. The obtained extract **E_5** was treated according to the procedure indicated in Scheme 3. In this procedure, **E_6** and **F_5** were characterized by TLC and ^{1}H-NMR spectroscopy.



Scheme 3. Schematic representation of extraction and purification procedure of Rock-spray leaves.

Extract **E_6** was obtained by liquid-liquid extraction with petroleum ether (green oil, 4%; m/m). TLC of **E_6** shows the presence of five compounds (R_f = 0.21, 0.31, 0.43, 0.72 and 0.84; DCM/Methanol, 9/1, v/v). The main features of ^{1}H-NMR are the presence of a doublet in the aromatic region at δ8.09 ppm (J = 7.2 Hz), a multiplet at δ7.63-7.59 ppm and a triplet at δ7.47 ppm (J = 8.0 Hz). There are also signals in the olefinic zone (δ5.40-3.82 ppm) and aliphatic zone (δ2.83-0.84 ppm).

Extract **E_6** was subjected to column chromatography, and a major fraction **F_5** was collected as a dark green oil, 1% (m/m); TLC of **F_5** shows the presence of three compounds (R_f = 0.44, 0.72 and 0.83; petroleum ether/DCM, 1/1, v/v). The ^{1}H-NMR spectra is similar to **E_6** with one major difference in aromatic zone, the appearance of two doublets at δ7.18 ppm (J = 7.6 Hz) and δ7.14 ppm (J = 7.2 Hz), instead of the pattern of signals previously described for **E_6**.

3.1.4 *Rosmarinus officinalis L.*

According to literature, rosemary essential oil has as its main constituents α-pinene **38** (up to 30%), β-pinene (2 to 6%), camphor **39** (15 to 25%), and in smaller amounts myrcene, *p*-cymene, borneol, verbenone **40**, varying the composition according to the time of year and the geographic region (Habtemariam, 2016). The fresh leaves of rosemary were subjected to hydrodistillation and an oil was obtained after liquid-liquid extraction (**E_7**). **E_7** was purified by dry-flash column chromatography and **F_6** (major fraction) was obtained and characterized by TLC, UV-Vis and ^{1}H-NMR spectroscopy.

TLC of **F_6** (colorless oil, 0.1%; m/m) shows the presence of a single compound (R_f = 0.52; ethyl acetate/DCM (1/1, v/v). The UV-Vis spectrum shows one band with maximum absorption at 249 nm (Figure 27). ^{1}H-NMR spectroscopy has confirmed the presence of verbenone **40** (Mills, 1996) (Figure 26). The main signals are a sextet at δ5.71 ppm corresponding to H-3; H-1, H-5 and one of the H-7 protons are shown as a triplet of doublets, triplet of doublets and doublet of triplets at δ2.65, 2.42 and 2.81 ppm, respectively. The methyl groups attached to C-6 arise as singlets at δ1.48 and 1.00 ppm respectively, while the CH_3 group attached to C-4 arises as a multiplet at δ2.01-2.03 ppm. The other H-7 proton is shown as a doublet at δ2.07 ppm (J = 9.2 Hz).

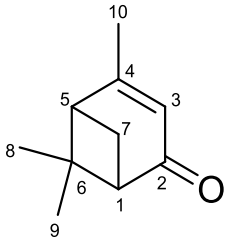


Figure 26. Structure of verbenone **40**.

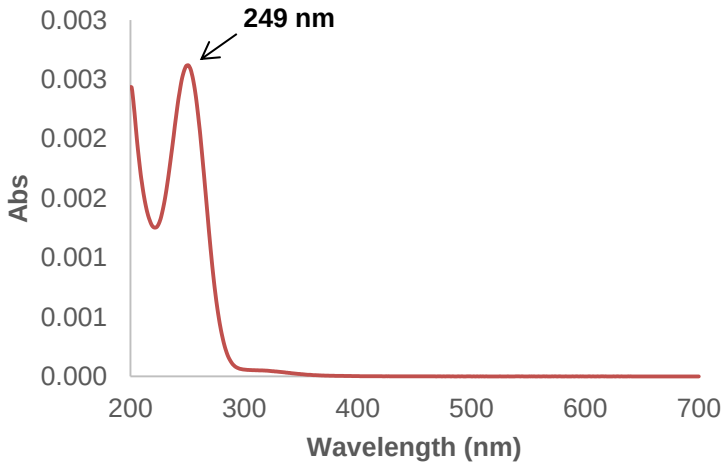
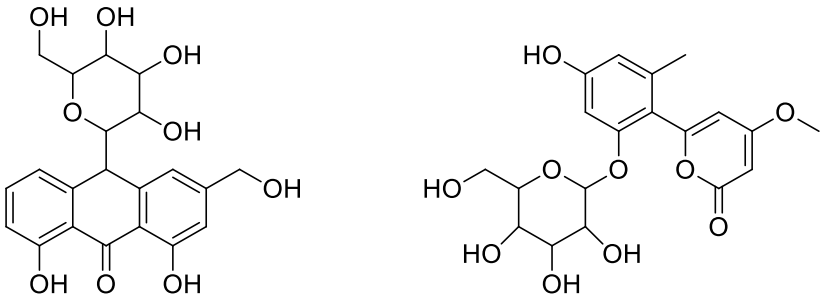


Figure 27. UV-Vis absorption spectrum of $\mathbf{F_6}$ (verbenone **40**).

3.1.5 *Aloe barbadensis Miller*

The fresh sap inside leaves of *Aloe Vera* was subjected to a liquid-liquid extraction. The extract obtained $\mathbf{E_8}$ was a blue solid and the ^{1}H-NMR spectrum suggests the existence of an anthraquinone derivative (Figure 28 **44**). Signals corresponding to the double bond protons (δ4.5-5.0 ppm) and the singlet relative to the RCHO group (δ9.9 ppm) along with characteristic zones of aromatic compounds resemble those shown in a spectrum ^{1}H NMR of the base structure of this class of compounds. UHPLC –ESI-TOF shows a complex mixture (Figure 29), which mass spectrum indicated the presence of compounds with molecular mass ranging from 114 to 555 m/z. ESI-TOF of compounds 4 and 5 present in the mixture indicated the presence of aloin **44** and

isomers (compound 4; HRMS 419.1327 m/z and compound 5; HRMS 419.1330 m/z) (Table 4). Compound 4 and 5 are probably aloin A and B, which are known to be the main biologically active constituents of Aloe Vera extracts. Aloin is a *C*-glycoside derivative of anthaquinone and is found in nature as a mixture of 2 diastereoisomers (Aloin A and B) (Celestino *et al.*, 2013). ESI-TOF of compound 2 also indicated the possibly presence of aloein **46** (compound 2; HRMS 411.1275 m/z).



44 **46**

Figure 28. Structure of aloin **44** and aloein **46.**

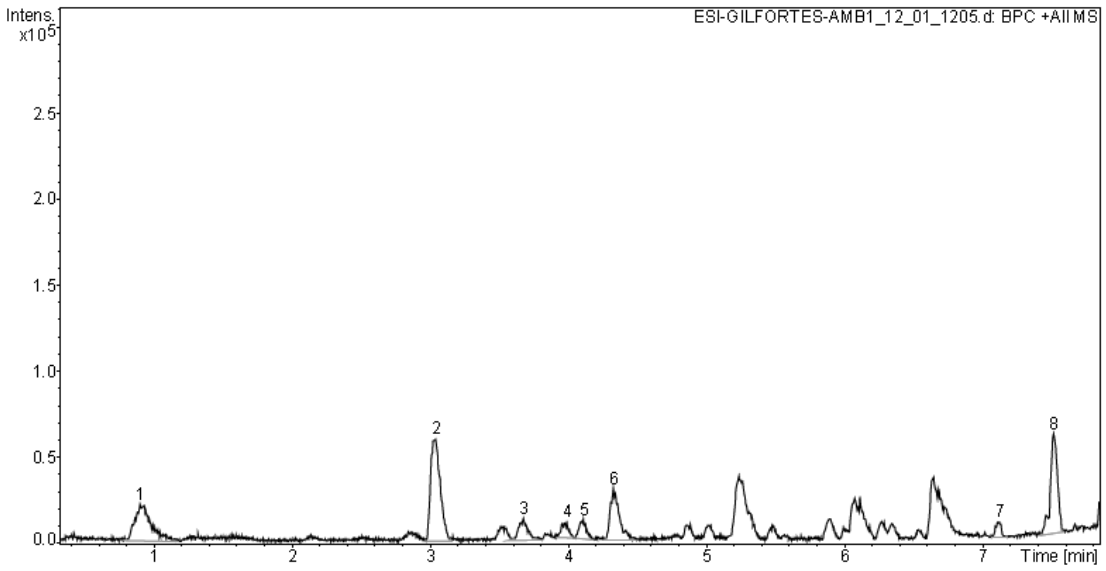


Figure 29. Chromatogram of extract (**E_8**) of *Aloe Vera* in UHPLC-ESI-TOF analysis.

Table 4. Measured and theoretical m/z for each major compounds identified by HRMS in $\mathbf{E_8}$.

#	Measured m/z	Ion formula	m/z
4	419.1327	$C_{21}H_{23}O_9$	419.1337
5	419.1330	$C_{21}H_{23}O_9$	419.1337

3.2 Cytotoxic activity of extracts obtained in human adenocarcinoma gastric of the stomach (AGS cell line)

Cancer annually accounts for 12 percent of total deaths worldwide, and in industrialized countries, 25 percent of people die of cancer each year. Gastric cancer is one of the most common cancers worldwide. A range of gastrointestinal cancers arise from inflammation and are preceded by a lengthy precancerous process, developing via multiple sequential steps. Stomach cancers are the second leading cause of cancer-related deaths, exhibiting a persistently high mortality (10.4% of all cancer deaths per year). To decrease gastric cancer mortality will require earlier diagnosis of these cancers, as well as a wider range of therapeutic alternatives (Carl-mcgrath *et al.*, 2007).

Plant extracts could be an alternative, as some of them exhibit anticancer activity through the induction of apoptosis or necrosis. Apoptosis is a physiological form of programmed cell death that is associated with chronic cell injury or the morphological result of a relatively slow process. It typically occurs in isolated individual cells within a tissue. In this type of cell death, cellular metabolism and membrane integrity are maintained until a very late stage of the process leading to apoptotic cell death. Morphological changes that occur during apoptosis include an early decrease in cell and nuclear volume, condensation and margination of chromatin at the nuclear periphery and nucleolar disintegration. Ultimately, apoptotic cells collapse and fragment into small membrane-encapsulated apoptotic bodies, which are phagocytosed by surrounding cells or macrophages without provoking an inflammatory response. Necrosis is often associated with acute toxic injury and in general rapidly affects tracts of contiguous cells in the damaged tissue. Necrotic tissue is characterized by cells exhibiting an increase in cell volume as a result of being unable to maintain ion homeostasis across the plasma membrane. Impairment of organelle function is an early

event, whereas random DNA degradation occurs late in this process due to the release of lysosomal enzymes from ruptured organelles (Manning et al., 2003).

P. americana L. (J. Das *et al.*, 2014), *T. patula L.* (Kashif *et al.*, 2015), *C. horizontalis D.* (Sokkar *et al.*, 2013), *R. officinalis L.* (Moore *et al.*, 2016) and *A. barbadensis M.* (Radha *et al.*, 2015) have been reported to exhibit anticancer properties. The cytotoxicity of triterpenoids saponins from *Phytolacca americana L.* was already evaluated in human ovarian cancer 2780 AD cells (Wang *et al.*, 2008). The cytotoxicity of amygdalin (which is one component present in Rock cotoneaster) was evaluated in MCF-7 breast cancer cell line (Shahrokhiyan *et al.*, 2018). The cytotoxicity of rosemary extracts was evaluated in human liver carcinoma cells (Vicente *et al.*, 2013), NCI-H82 human small cell lung carcinoma, DU-145 human prostate carcinoma cell, Hep-3B human black liver carcinoma cell, K-562 human chronic myeloid leukemia cell, MCF-7 human breast adenocarcinoma cell, PC-3 human prostate adenocarcinoma cell and MDA-MB-231 human breast adenocarcinoma cell (Yesil-Celiktas *et al.*, 2010). The cytotoxicity of aloe-emodin from *Aloe Vera* was already evaluated in human gastric carcinoma cells (AGS and NCI-N87 cell lines) (Chen *et al.*, 2007; Ghazanfari *et al.*, 2011) and in human liver cancer cell lines (Hep-G2 and Hep-3B) (Kuo *et al.*,2002).

The activity of the extracts obtained in this work against human adenocarcinoma gastric of the stomach cells (AGS cell line) was evaluated. To analyse the effect of these extracts on cell viability a MTT reduction assay was executed and to analyse the effect of these extracts on cell membrane integrity a lactate dehydrogenase (LDH) release assay was performed. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay is based on the metabolic potential, in this case, the mitochondrial enzyme activity of the cells (Figure 30). Cells with active metabolism convert MTT to formazan, with a purple color and a maximum absorption of 570 nm. When cells die, they lose the ability to convert MTT and thus, color formation serves as an indicator of cell viability *(Zou et al.*, 2013).

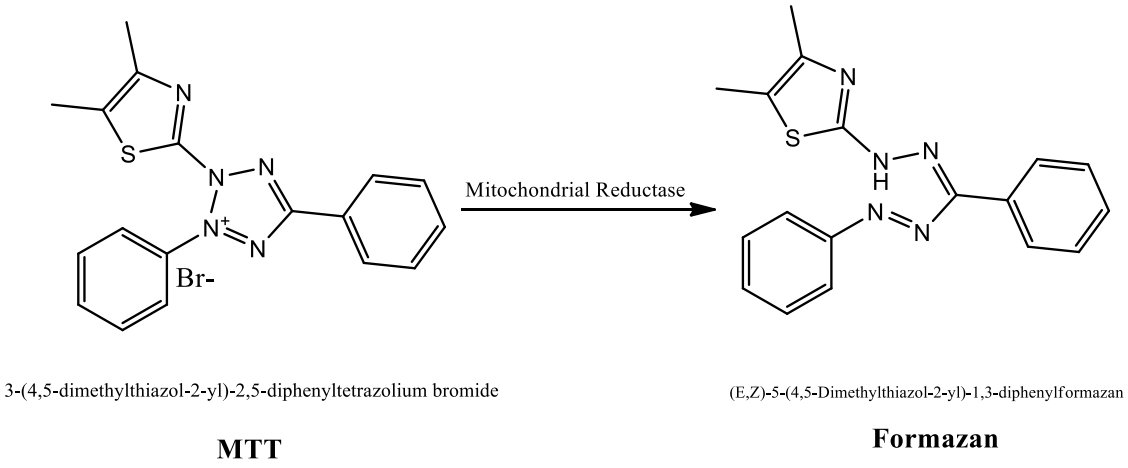


Figure 30. Mitochondrial reduction of MTT to formazan.

The lactate dehydrogenase (LDH) release assay is a method to analyze the cell membrane integrity, so the release of intracellular substances into the medium serves as an indicator of this integrity. LDH is a cytosol marker enzyme and frequently used to stimulate cell membrane integrity (Figure 31). So LDH (present in medium) is used in pyruvate→Lactate reaction (Canvax Biotech, 2017; Zou et al., 2013).

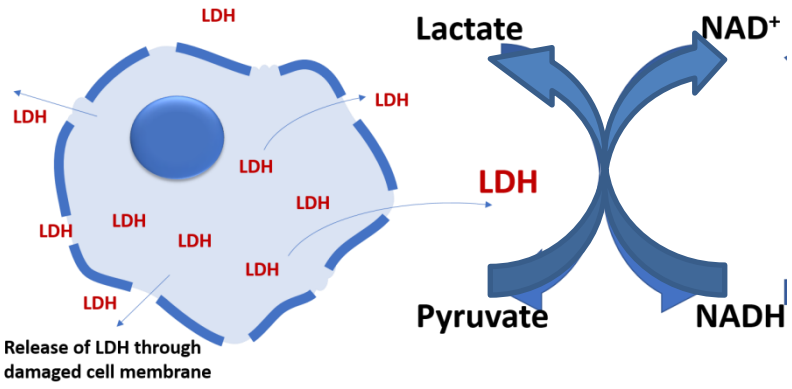


Figure 31. Schematic representation of the reduction of NADH to NAD^+ by the LDH enzyme with lactate formation (Canvax Biotech, 2017).

With these assays, it is possible to assume the IC_{50} of these extracts against human adenocarcinoma gastric of the stomach cells (AGS cell line). IC_{50} is the dose of the extract required to kill 50% of cancer cells.

In *P. Americana L.*, the effect of the extract (**E_1**) and the reaction mixture of hydrolysis (**E_2**) on cell viability were analysed. The reaction mixture (**E_2**) shows more toxicity, with an IC_{50} of 0.25 mg/mL, than extract (**E_1**), which at a concentration of 1mg/mL presents ~75% of cell viability. The effect of these two samples on cell membrane integrity was also analysed. The reaction mixture (**E_2**) shows positive LDH at the highest concentrations; *i.e.* LDH is released to the cytosol due to membrane rupture which means that probably necrosis occurs. The extract (**E_1**) does not cause the release of LDH to the cytosol at any of the concentrations tested, so the toxicity that this extract shows is not related to processes leading to cell necrosis (Figure 32).

In *T. patula L.*, the effect of the three fractions (**F_1**, **F_2** and **F_3**) obtained previously on cell viability and cell membrane integrity were studied. All fractions cause toxicity in the highest concentrations, in which the most toxic fraction is fraction 2 (**F_2**) of aqueous extraction, with IC_{50} of 0.5 mg/mL. In the LDH release assay, none of the fractions have shown to cause LDH release to the cytosol for any of the concentrations tested, so the toxicity of these fractions does not appear to be related to processes leading to cell necrosis (Figure 32).

In addition, the effect of extract (**E_6**) and fraction (**F_5**) of *C. cotoneaster D.* on cell viability and cell membrane integrity was investigated. Extract (**E_6**) and fraction (**F_5**) have shown toxicity at high concentrations, in which the extract (**E_6**) presents higher toxicity, with IC_{50} of 1mg/mL. **E_6** and **F_5** show negative LDH release assay, which indicates that toxicity of these samples is not related to the processes leading to cell necrosis (Figure 33).

Toxicity of the *R. officinalis L.* fraction (**F_6**, verbenone **40**) and the *A. barbadensis M.* extract (**E_8**) were also evaluated by the MTT reduction assay and the LDH release assay. Both samples (**F_6** and **E_8**) demonstrated toxicity at high concentrations, in which verbenone **40** showed higher toxicity with IC_{50} of 1 mg/mL. In LDH release assay, the verbenone **40** presented LDH positive in the highest concentrations, indicating that LDH release to the cytosol and the cellular membrane is ruptured, evidencing that necrosis occurred; *A. barbadensis M.* extract **E_8** showed LDH negative indicating that the toxicity of this sample does not appear to be related to processes leading to cell necrosis (Figure 33).

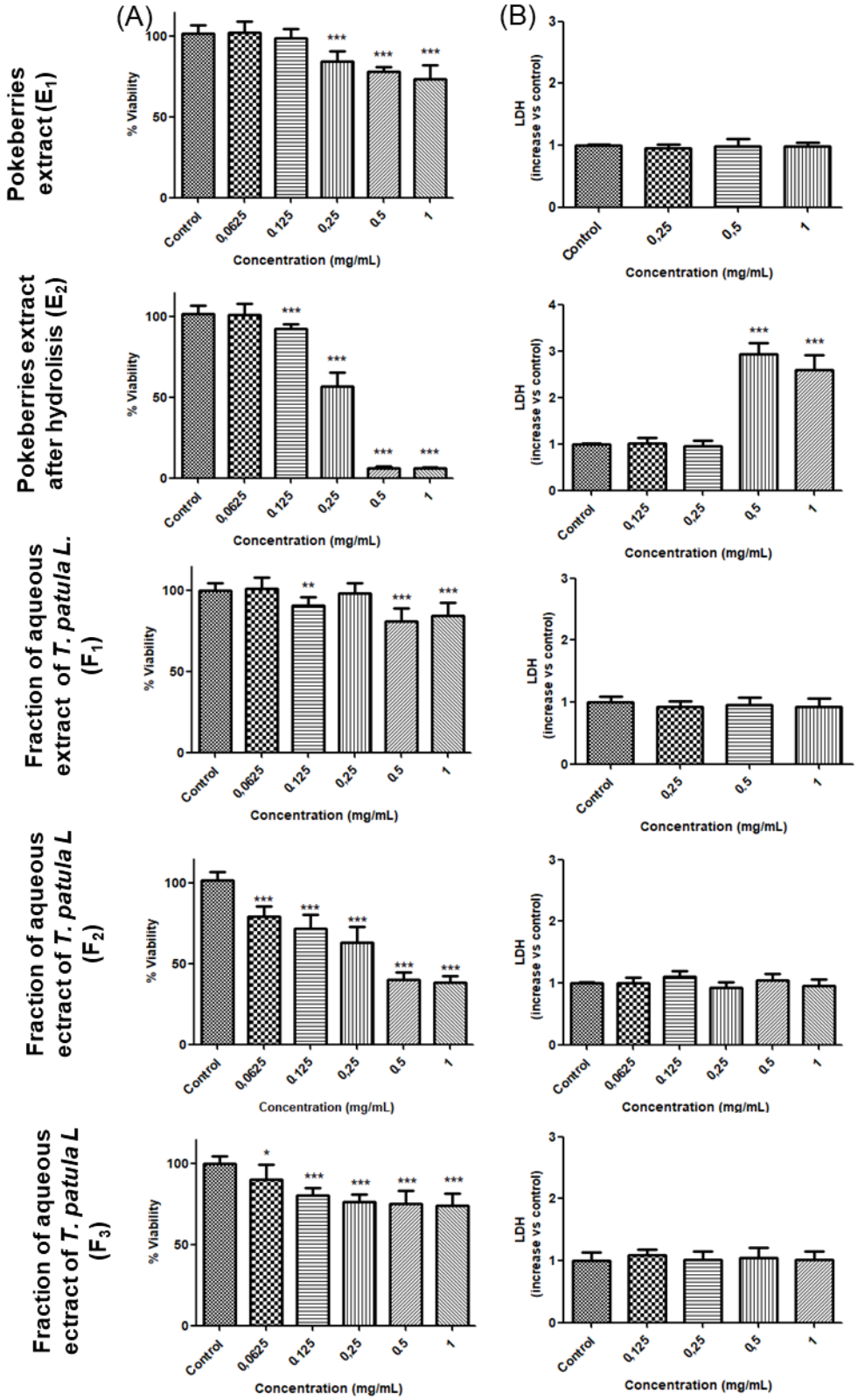


Figure 32. Effect of extracts/fractions obtained in Pokeberries and French marigold species on viability (A) and cell membrane integrity (B) on the AGS cell line. Cells (density $1x10^4$) were incubated with the different concentrations of extracts/fractions at 37 ° C for 24 hours. Data represent the mean ± standard deviation of the mean of five concentrations, in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the respective control.

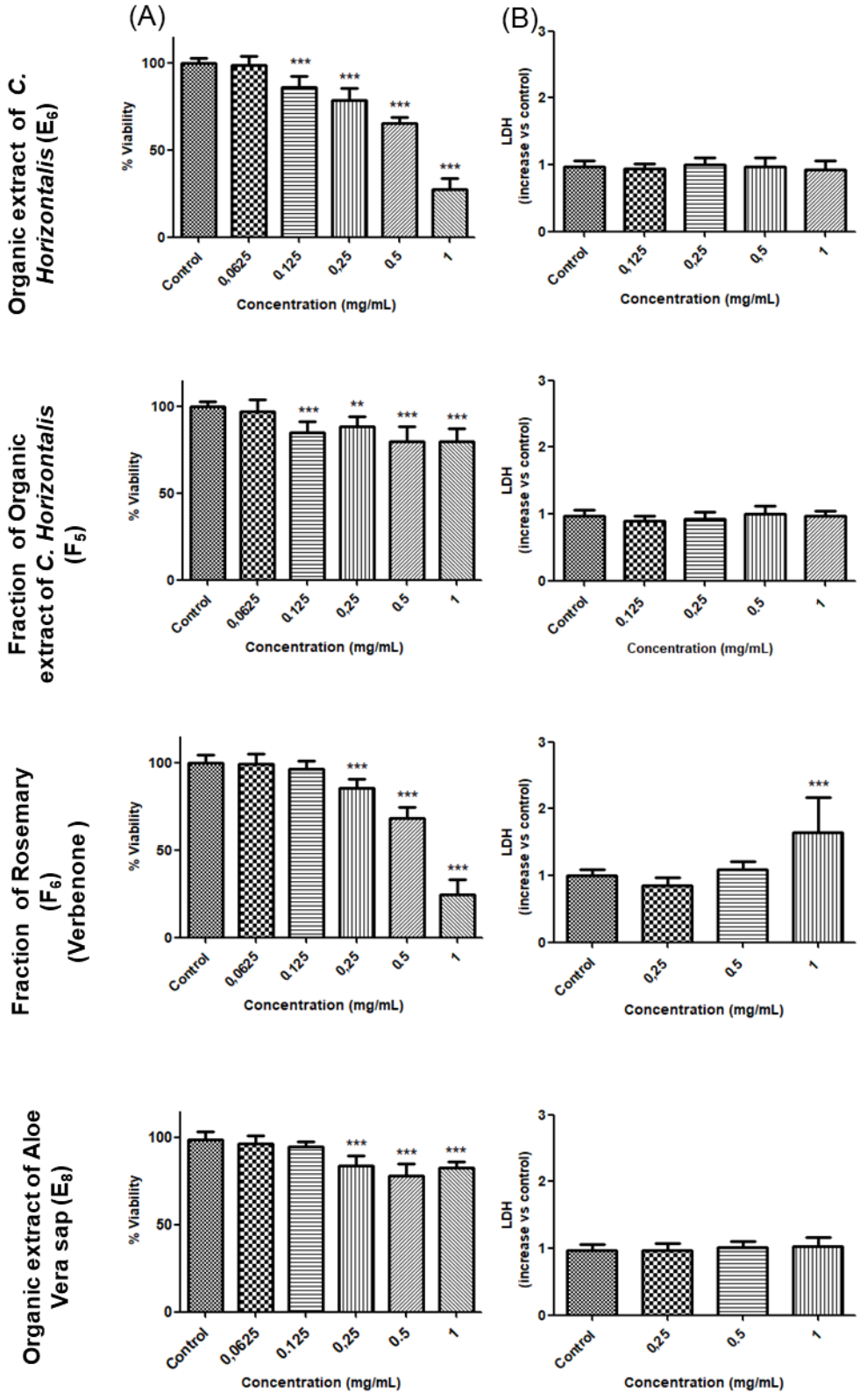


Figure 33. Effect of extracts/fractions obtained in Rock cotoneaster, Rosemary and Aloe Vera species on viability (A) and cell membrane integrity (B) on the AGS cell line. Cells (density $1x10^4$) were incubated with the different concentrations of extracts/fractions at 37 ° C for 24 hours. Data represent the mean ± standard deviation of the mean of five concentrations, in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the respective control.

3.3 Encapsulation of extracts of *P. americana L.* and *T. patula L.* in liposomes

As previously stated, the encapsulation of bioactive compounds increases the physical stability, modulates their release, protects from oxidation reaction with environment, decreases volatility and enhances bioactivity (Bilia *et al.*, 2014).

In previous studies, an ethanolic extract of *P. decandra* in which a triterpenoid was identified, was encapsulated in biodegradable nontoxic polymers of poly (lactide-co-glycolide). The effect of non-encapsulated and encapsulated extract in the viability in human non small cell lung carcinoma (A549 cells) was studied. The results showed that the encapsulated extract has a stronger cytotoxic effect (with IC_{50} of 50 µg/mL), than the non-encapsulated extract (with IC_{50} of 100 µg/mL) (J. Das *et al.*, 2014). So, the extracts obtained (**E_1** and **E_2**) of *P. americana L.* were encapsulated in a commercial lipid mixture (soybean lecithin) to evaluate, in future, if the encapsulated extract could produce enhanced anticancer effects. The method of ethanolic injection was used for the incorporation of extracts in the lipid-based nanosystems. The choice of the natural and economical soybean lecithin was motivated by the fact that this lipid mixture has already shown a good potential in the encapsulation of textile dyestuffs for dyeing (Baptista *et al.*, 2004; De Sousa *et al.*, 2011). Moreover, the ethanolic injection method has allowed to obtain high encapsulation efficiencies of hydrophobic/lipophilic compounds (Jaafar-Maalej et al., 2010). Previously, a calibration curve of absorbance *versus* concentration was obtained and linearity was demonstrated. Therefore, the concentration of the encapsulated extract was determined by the Beer-Lambert law (equation 1) shown in section 2.7. The encapsulation efficiency was determined by equation 2 and the results are presented in Table 5 for the two extracts used. The nanoencapsulation assay showed a high encapsulation efficiency (88.6%) for the extract of pokeweed before hydrolysis reaction (**E_1**), while after hydrolysis (**E_2**), the efficiency was low (28.2%). These results can be due to the different hydrophobicity of both extracts, as the method used is very suitable for lipophilic compounds, but has shown a low efficiency for hydrophilic ones (Jaafar-Maalej et al., 2010). For **E_2** extract, the thin film hydration method could be an alternative for the same lipid formulation. This technique, as well as other lipid formulations, will be explored in the near future.

Table 5. Results obtained through the encapsulation of the $\mathbf{E_1}$ and $\mathbf{E_2}$ extracts.

Samples	Wavelength (nm)	ε ($M^{-1}cm^{-1}$)	Encapsulation efficiency (%)
E_1	400	158	88.6
E_2	280	1738	28.2

As far as we know, only the marigold flowers are commercially cultivated as a source of lutein **28**. Lutein is a carotenoid, which presents anticancer properties; however, this compound is an unstable molecule, with low bioavailability due to its insolubility in aqueous medium. In a previous study (Brum *et al.*, 2017), lutein nanocapsules were developed by the technique of interfacial deposition of the preformed polymer poly-$\mathcal{E}$-caprolactone (PCL). This nanoencapsulation assay allowed the solubilisation of this carotenoid in aqueous medium and increased the stability of lutein in different temperatures. Furthermore, lutein nanocapsules presented 43% more biological activity than the free lutein. So, the fraction obtained $\mathbf{F_4}$ of *T. patula L*, which suggests the presence of carotenoids. was encapsulated in a commercial lipid mixture (soybean lecithin) to evaluate, in future, if the encapsulated fraction in liposomes could produce enhanced anticancer effects. The method of ethanolic injection was also used and the procedure was described above in section 2.7. The nanoencapsulation assay showed a high encapsulation efficiency (98.1%) for the fraction $\mathbf{F_4}$ of French marigold (Table 6).

Table 6. Results obtained through the encapsulation of the $\mathbf{F_4}$ fraction.

Sample	Wavelength (nm)	ε ($M^{-1}cm^{-1}$)	Encapsulation efficiency (%)
F_4	470	2667	98.1

Chapter 4: Conclusion and future perspectives

Plants have a high phytochemical diversity in which many of them possess interesting potential biological activities. However, some phytochemicals are extremely unstable which could be a problem for potential applications. *Phytolacca americana L.*, *Tagete patula L.*, *Cotoneaster horizontalis Decne*, *Rosmarinus officinalis L.* and *Aloe barbadensis Miller* were chosen to extract and characterise their phytochemicals and also to evaluate their anticancer potential. Some extracts were encapsulated using lipid-based nanosystems.

Extracts characterization of pokeweed suggests the presence of saponins in $\mathbf{E_1}$ and sapogenins in $\mathbf{E_2}$ (after hydrolysis). Both extracts have shown toxicity against human adenocarcinoma gastric of the stomach cells, with $\mathbf{E_2}$ showing the highest toxicity which can be related to processes leading to cell necrosis. The encapsulation of these extracts in lipid nanosystems was also carried out and the extract $\mathbf{E_1}$ displayed better encapsulation efficiency.

French marigold extraction afforded $\mathbf{F_1}$, $\mathbf{F_2}$, $\mathbf{F_3}$ and $\mathbf{F_4}$ fractions. Their characterization suggested the presence of terpenes, terpenoids and essential oils ($\mathbf{F_1}$ and $\mathbf{F_2}$), phenolic compounds ($\mathbf{F_3}$) and carotenoids ($\mathbf{F_4}$). The toxicity of the $\mathbf{F_1}$, $\mathbf{F_2}$ and $\mathbf{F_3}$ was evaluated against AGS cells. All these fractions cause toxicity in highest concentrations and none of them appear to be related to process leading to cell necrosis. $\mathbf{F_4}$ was also encapsulated in lipid nanosystems and it showed high encapsulation efficiency.

Extract $\mathbf{E_6}$ and fraction $\mathbf{F_5}$ of rock cotoneaster were obtained and analysed. Although it was not possibly to identify their composition in detail, anticancer potential of these samples was evaluated and both showed toxicity at high concentrations not related to the processes leading to cell necrosis.

One fraction $\mathbf{F_6}$ of rosemary identified as verbenone was also evaluated against AGS cells showing toxicity and indicating that necrosis occurred.

Characterization of extract $\mathbf{E_8}$ from *Aloe vera* suggested the presence of anthraquinone type compounds. This extract also caused toxicity against AGS cells at high concentrations, but it is not related to processes leading to cell necrosis.

Future studies should include the isolation and full characterization of these extracts/fractions composition. In the case of biological assays, the toxicity of these extracts/fractions obtained should be evaluated in non-cancer cells to evaluate their specificity, *i.e.*, whether these extracts are only toxic to cancer cells. In the case of encapsulation assays, the formulations obtained should be characterized, for example by

their particle size, polydispersity index and stability, besides the determination of encapsulation efficiency. Other lipid nanosystems should also be evaluated, as well as the thin film hydration method for the preparation of liposomes. Biological activity of encapsulated extracts/compounds will be carried out, in order to compare their potential with non-encapsulated materials.

Chapter 5: References

Abdel-Aal, E. S. M., Rabalski, I. (2015). Composition of Lutein Ester Regioisomers in Marigold Flower, Dietary Supplement, and Herbal Tea. *J. Agric. Food. Chem.*, *63*(*44*), 9740–9746.

Alves, D. S., Pérez-Fons, L., Estepa, A., Micol, V. (2004). Membrane-related effects underlying the biological activity of the anthraquinones emodin and barbaloin. *Biochem. Pharmacol.*, *68*(*3*), 549–561.

Ateeq, M., Shah, M. R., Ain, N. U., Bano, S., Anis, I., Lubna, Sohaila Naz, S. (2015). Green synthesis and molecular recognition ability of patuletin coated gold nanoparticles. *Biosens. Bioelectron.*, *63*, 499–505.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M. (2008). Biological effects of essential oils - A review. *Food Chem. Toxicol.*, *46*(*2*), 446–475.

Balasundram, N., Sundram, K., Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.*, *99*(*1*), 191–203.

Banu, K. S., & Cathrine, L. (2015). General Techniques Involved in Phytochemical Analysis. *Int.J. of Adv Res. in Chem. Sci.*, *2*(*4*), 25–32.

Baptista, A. L. F., Coutinho, P. J. G., Oliveira, M. E. C. D. R., Gomes, J. I. N. R. (2004). Lipid interaction with textile fibres in dyeing conditions. *Colloid Polym. Sci.*, 88–93.

Bhattacharyya, S., Datta, S., Mallick, B., Dhar, P., Ghosh, S. (2010). Lutein content and in vitro antioxidant activity of different cultivars of Indian marigold flower (Tagetes patula l.) extracts. *J. Agric. Food. Chem.*, *58*(*14*), 8259–8264.

Bilia, A. R., Guccione, C., Isacchi, B., Righeschi, C., Firenzuoli, F., Bergonzi, M. C. (2014). Essential oils loaded in nanosystems: A developing strategy for a successful therapeutic approach. *Evidence-Based Complementary and Alternative Med.*, *2014*.

Brum, A. A. S., Santos, P. P. dos, Silva, M. M. da, Paese, K., Guterres, S. S., Costa, T. M. H., Rios, A. de O. (2017). Lutein-loaded lipid-core nanocapsules: Physicochemical characterization and stability evaluation. *Colloids and Surf. A:*

Physicochemical and Eng. Aspects, *522*, 477–484.

Canvax Biotech, S. L. (2017). LDH cytotoxicity assay Kit.

Carl-mcgrath, S., Ebert, M., Röcken, C. (2007). Gastric adenocarcinoma : epidemiology , pathology and pathogenesis Review Article. *Cancer Therapy*, *5* (2007), 877–894.

Celestino, V. R. L., Maranhão, H. M. L., Vasconcelos, C. F. B., Lima, C. R., Medeiros, G. C. R., Araújo, A. V., & Wanderley, A. G. (2013). Acute toxicity and laxative activity of Aloe ferox resin. *Brazilian Journal of Pharmacognosy*, *23*(2), 279–283.

Chen, S., Lin, K., Chang, C., Fang, C., Lin, C. (2007). Aloe-emodin-induced apoptosis in human gastric carcinoma cells. *Food Chem. Toxicol.* 45(11) 2296-2303.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, *12*(4), 564–582.

Crozier, A., Jaganath, I. B., Clifford, M. N. (2009). Dietary phenolics: Chemistry, bioavailability and effects on health. *Clin. Microbiol. Rev.*, *26*(8), 1001–1043.

Cumpson, P., Sano, N. (2013). Stability of reference masses V: UV/ozone treatment of gold and platinum surfaces. *Metrologia*, *50*(1), 27–36.

Cushnie, T. P. T., Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, *26*(5), 343–356.

Silva, D. C., Andrade, P. B., Valentão, P., Pereira, D. M. (2017). Neurotoxicity of the steroidal alkaloids tomatine and tomatidine is RIP1 kinase- and caspase-independent and involves the eIF2α branch of the endoplasmic reticulum. *J. Steroid Biochem. Mol. Biol.*, *171*, 178–186.

Dai, J., Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Mol.*, *15*(10), 7313–7352.

Das, J., Das, S., Paul, A., Samadder, A., Khuda-Bukhsh, A. R. (2014). Strong Anticancer Potential of Nano-triterpenoid from Phytolacca decandra against A549 Adenocarcinoma via a Ca2+-dependent Mitochondrial Apoptotic Pathway. *JAMS Journal of Acupuncture and Meridian Stud.*, *7*(3), 140–150.

Das, K., Tiwari, R. K. S., Shrivastava, D. K. (2010). Techniques for evaluation of

medicinal plant products as antimicrobial agents: current methods and future trends. *J. Med. Plants Res.*, *4*(2), 104–111.

De Sousa, I. S. C., Castanheira, E. M. S., Rocha Gomes, J. I. N., Real Oliveira, M. E. C. D. (2011). Study of the release of a microencapsulated acid dye in polyamide dyeing using mixed cationic liposomes. *J. Liposome Res.*, *21*(2), 151–157.

Ding, L. J., Ding, W., Zhang, Y. Q., Luo, J. X. (2013). Bioguided fractionation and isolation of esculentoside P from Phytolacca americana L. *Ind. Crops Prod.*, *44*, 534–541.

Dolara, P., Luceri, C., De Filippo, C., Femia, A. Pietro, Giovannelli, L., Caderni, G., Cresci, A. (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutation Res. Fundam. Mol. Mechanisms Mutagenesis*, *591*(1–2), 237–246.

El-baz, F. K., Mahmoud, K., El-senousy, W. M., Darwesh, O. M., Elgohary, A. E. (2015). Eucalyptus camaldulensis Essent. Oils, *31*(50), 262–268.

Escalante, A. M., Santecchia, C. B., López, S. N., Gattuso, M. A., Gutiérrez Ravelo, A., Delle Monache, F., Zacchino, S. A. (2002). Isolation of antifungal saponins from Phytolacca tetramera, an Argentinean species in critic risk. *J. Ethnopharmacol.*, *82*(1), 29–34.

Eze, U. A. (2016). In vitro Antimicrobial Activity of Essential Oils from the Lamiaceae and Rutaceae Plant Families Against β-Lactamse-Producing Clinical Isolates Of Moraxella Catarrhalis. *EC* Pharm. Sci., *2.3*, 325–337.

Faizi, S., Fayyaz, S., Bano, S., Yawar Iqbal, E., Lubna, L., Siddiqi, H., & Naz, A. (2011). Isolation of nematicidal compounds from tagetes patula L. yellow flowers: Structure-activity relationship studies against cyst nematode heterodera zeae infective stage larvae. *J. Agric. Food Chem.*, *59*(17), 9080–9093.

Fang, Z., Bhandari, B. (2010). Encapsulation of polyphenols - A review. *Trends Food Sci. Technol.*, *21*(10), 510–523.

Ferhat, M. A., Meklati, B. Y., Chemat, F. (2007). Comparison of different isolation

methods of essential oil from Citrus fruits: cold pressing, hydrodistillation and microwave 'dry' distillation. *Flavour Fragrance J.*, *22*(November), 206–213.

Garg, S. N., Charles, R., Kumar, S. (1999). A new acyclic monoterpene glucoside from the capitula of Tagetes patula. *Fitoterapia*, *70*(5), 472–474.

Ghazanfari, T., Yaraee, R., Shams, J., Rahmati, B., Radjabian, T., Hakimzadeh, H. (2013). Cytotoxic effect of four herbal medicines on gastric cancer (AGS) cell line. *Food Agric. Immunol.*, 24(1), 1–7.

Gillij, Y. G., Gleiser, R. M., Zygadlo, J. A. (2008). Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. Bioresour. Technol., *99*(7), 2507–2515.

Greathead, H. (2003). Plants and plant extracts for improving animal productivity. *Proc. Nut. Soc.*, *62*(02), 279–290.

Habtemariam, S. (2016). The Therapeutic Potential of Rosemary (Rosmarinus officinalis) Diterpenes for Alzheimer's Disease. *Evid. Complem. Alternative Med.*, *2016*.

Harbone, J., Tomas-barberan, F. (1991). Ecological Chemistry and Biochemistry of Plant Terpenoids . *Clarendon, Oxford*, *9*(S1), 169–174.

Iglesias, R., Citores, L., Ragucci, S., Russo, R., Di Maro, A., Ferreras, J. M. (2016). Biological and antipathogenic activities of ribosome-inactivating proteins from Phytolacca dioica L. *Biochim. Biophys. Acta- General Subjects*, *1860*(6), 1256–1264.

Jaafar-Maalej, C., Diab, R., Andrieu, V., Elaissari, A., Fessi, H. (2010). Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. *J. Liposome Res.*, *20*(3), 228–243.

Jabeen, A., Mesaik, M. A., Simjee, S. U., Lubna, Bano, S., Faizi, S. (2016). Anti-TNF-α and anti-arthritic effect of patuletin: A rare flavonoid from Tagetes patula. *Int. Immunopharmacol.*, *36*, 232–240.

Jerz, G., Skotzki, T., Fiege, K., Winterhalter, P., Wybraniec, S. (2008). Separation of betalains from berries of Phytolacca americana by ion-pair high-speed counter-

current chromatography. *J. Chromatogr. A*, *1190*(1–2), 63–73.

Kashif, M., Bano, S., Naqvi, S., Faizi, S., Lubna, Ahmed Mesaik, M., Farooq, A. D. (2015). Cytotoxic and antioxidant properties of phenolic compounds from Tagetes patula flower. *Pharm. Biol.*, *53*(5), 672–681.

Kennedy, D. O. Do, Wightman, E. E. L. (2011). Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Adv. Nut. An* , *2*(August), 32–50.

Khan, S., Runguo, W., Tahir, K., Jichuan, Z., Zhang, L. (2017). Catalytic reduction of 4-nitrophenol and photo inhibition of Pseudomonas aeruginosa using gold nanoparticles as photocatalyst. *J. Photochem. Photobiol. B: Biol.*, *170*, 181–187.

Khan, S., Wang, Z., Wang, R., Zhang, L. (2014). Horizontoates A-C: New cholinesterase inhibitors from Cotoneaster horizontalis. *Phytochem. Lett.*, *10*, 204–208.

Klaunig, J. E., Wang, Z., Pu, X., Zhou, S. (2011). Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol. Appl Pharm.*, *254*(2), 86–99.

Kremer, J. M. H., Esker, M. W. J. V. D., Pathmamanoharan, C., Wiersema, P. H. (1977). Vesicles of Variable Diameter Prepared by a Modified Injection Method. *Biochem.*, *16*(17), 3932–3935.

Kuo, P., Lin, T., Lin, C. (2002). The antiproliferative activity of aloe-emodin is through P53-dependent and P21-dependent apoptotic pathway in human hepatoma cell lines. *Life Sci.* 71(16), 1879-18-92.

Kurmukov, A. G. (2013). Phytochemistry of medicinal plants. *Med. Plants Central Asia: Uzbekistan and Kyrgyzstan*, *1*(6), 13–14.

Lacaille-Dubois, M. A., Wagner, H. (2000). Bioactive saponins from plants: An update. *Stud. Nat. Prod. Chem.*, *21*(PART B), 633–687.

Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C., Fessi, H. (2012). Preparation, Characterization and Applications of Liposomes: State of the Art. *J. Colloid Sci. Biotechnol.*, *1*(2), 147–168.

Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X., Deng, Y. (2014). A review on phospholipids and their main applications in drug delivery systems. *Asian J. Pharm. Sci.*, *10*(2), 81–98.

Lubbe, A., Verpoorte, R. (2011). Cultivation of medicinal and aromatic plants for specialty industrial materials. *Ind. Crops Prod.*, *34*(1), 785–801.

M. Saxena, J. Saxena, R. Nema, D. Singh, A. G. et al. (2013). Phytochemistry of Medicinal Plants - ProQuest. *J. Pharmacog. Phytochem.*, *1*(6), 168–182.

Manning, F., Zuzel, K. (2003). Comparison of types of cell death: Apoptosis and necrosis. *J. Biol. Edu.*, *37*(3), 141–145.

Mills, N. S. (1996). Complete Assignment of Proton Chemical Shifts in Terpenes: An Experiment Combining 2D NMR Spectroscopy with Molecular Modeling. *J. Chem. Edu.*, *73*(12), 1190.

Mohamed, S. A., Sokkar, N. M., El-Gindi, O., Ali, Z. Y., Alfishawy, I. M. (2012). Phytoconstituents Investigation, Anti-diabetic and Anti-dyslipidemic Activities of Cotoneaster horizontalis Decne Cultivated in Egypt, *9*, 394–403.

Moore, J., Yousef, M., & Tsiani, E. (2016). Anticancer effects of rosemary (Rosmarinus officinalis L.) extract and rosemary extract polyphenols. *Nut.* (Vol. 8).

Moyler, D. A. (1993). Extraction of Essential Oils with Carbon Dioxide. *Flav. Fragan. J.*, *8*(January), 235–247.

Ncube, N. S., Afolayan, A. J., Okoh, A. I. (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African J. Biotechnol.*, *7*(12), 1797–1806.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *Int. J. Food Microbiol.*, *156*(1), 7–17.

Oliveira, A. P., Silva, L. R., Andrade, P. B., Valentao, P., Silva, B. M., Gonc-Alves, R. F., De Pinho, P. G. (2010). Further insight into the latex metabolite profile of ficus carica. *J. Agric. Food Chem.*, *58*(20), 10855–10863.

Özcan, M. M., Chalchat, J. C. (2008). Chemical composition and antifungal activity of

rosemary (Rosmarinus officinalis L.) oil from Turkey. *Int. J. Food Sci. Nut.*, *59*(7–8), 691–698.

Pedrós, R., Moya, I., Goulas, Y., Jacquemoud, S. (2008). Chlorophyll fluorescence emission spectrum inside a leaf. *Photochem. Photobiol. Sci.*, *7*(4), 498–502.

Petit-Paly, G., Andreu, F., Chénieux, J. C., Rideau, M. (1994). Phytolacca americana L. (Pokeweed): In Vitro Production of Betacyanins and Medicinal Compounds. In *Biotechnol. Agric. Forestry* (Vol. 28, pp. 366–385).

Politi, F. A. S., De Souza-Moreira, T. M., Rodrigues, E. R., De Queiroz, G. M., Figueira, G. M., Januário, A. H., Pietro, R. C. L. R. (2013). Chemical characterization and acaricide potential of essential oil from aerial parts of Tagetes patula L. (Asteraceae) against engorged adult females of Rhipicephalus sanguineus (Latreille, 1806). *Parasitol. Res.*, *112*(6), 2261–2268.

Politi, F. A. S., Figueira, G. M., Araújo, A. M., Sampieri, B. R., Mathias, M. I. C., Szabó, M. P. J., Linhari Rodrigues Pietro, R. C. (2012). Acaricidal activity of ethanolic extract from aerial parts of Tagetes patula L. (Asteraceae) against larvae and engorged adult females of Rhipicephalus sanguineus (Latreille, 1806). *Parasit. Vect.*, *5*(1), 295.

Prakash, O., Rout, P. K., Chanotiya, C. S., Misra, L. N. (2012). Composition of essential oil, concrete, absolute and SPME analysis of Tagetes patula capitula. *Ind. Crops Prod.*, *37*(1), 195–199.

Prashant Tiwari, B., Kumar, M. K., Gurpreet Kaur, H. K. (2011). Phytochemical screening and extraction - A review. *Int. Pharm. Sci.*, *1*(1), 98–106.

Proestos, C., Boziaris, I. S., Nychas, G. J. E., Komaitis, M. (2006). Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chem.*, *95*(4), 664–671.

Radha, M. H., Laxmipriya, N. P. (2015). Evaluation of biological properties and clinical effectiveness of Aloe vera: A systematic review. *J. Trad. Complemen. Med.*, *5*(1), 21–26.

Raksha, B. (2014). Bioactive Compounds and Medicinal Properties of Aloe Vera L.:

An Update. *J. Plant Sci. (Science Publishing Group)*, *2*(3), 102.

Rao, N. (2003). Bioactive phytochemicals in Indian foods and their potential in health promotion and disease prevention. *Asia Pacific J. Clin. Nut.*, *12*(1), 9–22.

Rodríguez, J., Martín, M. J., Ruiz, M. A., Clares, B. (2016). Current encapsulation strategies for bioactive oils: From alimentary to pharmaceutical perspectives. *Food Res. Int.l*, *83*, 41–59.

Romanik, G., Gilgenast, E., Przyjazny, A., Kamiński, M. (2007). Techniques of preparing plant material for chromatographic separation and analysis. *J. Biochem. Biophys. Methods*, *70*(2), 253–261.

Russo, G. L. (2007). Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochem. Pharmacol.* , *74*(4), 533–544.

Sahraoui, N., Vian, M. A., Bornard, I., Boutekedjiret, C., Chemat, F. (2008). Improved microwave steam distillation apparatus for isolation of essential oils. Comparison with conventional steam distillation. *J. Chromatography A*, *1210*(2), 229–233.

Samadi, M., Abidin, Z. Z., Yunus, R., Awang Biak, D. R., Yoshida, H., Lok, E. H. (2017). Assessing the kinetic model of hydro-distillation and chemical composition of Aquilaria malaccensis leaves essential oil. *Chinese J. Chem. Eng.*, *25*(2), 216–222.

Sampath, V., Golla, K., Sharabu, R. (2017). of Biomedical AND Pharmaceutical sciences Antioxidat studies of aqueous methanolic extract of canthium, *4*(12), 862–866.

Scala, K. Di, Vega-Gálvez, A., Ah-Hen, K., Nuñez-Mancilla, Y., Tabilo-Munizaga, G., Pérez-Won, M., Giovagnoli, C. (2013). Chemical and physical properties of aloe vera (Aloe barbadensis Miller) gel stored after high hydrostatic pressure processing. *Food Sci. Technol. Campinas)*, *33*(1), 52–59.

Shahrokhiyan, I., Doosti, A., Sazegar, H. (2018). Evaluation of the effect of amygdalin on survivin gene expression in MCF-7 breast cancer cell line. *Genetika* 50(2), 647-657.

Sherry, M., Charcosset, C., Fessi, H., Greige-Gerges, H. (2013). Essential oils

encapsulated in liposomes: A review. *J. Liposome Res.*, *23*(4), 268–275.

Shindo, K., Kikuta, K., Suzuki, A., Katsuta, A., Kasai, H., Yasumoto-Hirose, M., Takaichi, S. (2007). Rare carotenoids, (3R)-saproxanthin and (3R,2′S)-myxol, isolated from novel marine bacteria (Flavobacteriaceae) and their antioxidative activities. *Appl. Microbiol. Biotechnol.*, *74*(6), 1350–1357.

Siegel, R. A., Rathbone, M. J. (2012). Fundamentals and Applications of Controlled Release Drug Delivery, 19–43.

Slabaugh, P. E., Shaw, N. L. (1990). Cotoneaster. *Forest. Sci.*, (1986), 442–446.

Sokkar, N., El-Gindi, O., Sayed, S., Mohamed, S., Ali, Z., Alfishawy, I. (2013). Antioxidant, anticancer and hepatoprotective activities of Cotoneaster horizontalis Decne extract as well as a-tocopherol and amygdalin production from in vitro culture. *Phys Lett*, *35*, 2421–2428.

Sotelo, F. F., Pantoja, P. A., López-Gejo, J., Roux, G. A. C. Le, Quina, F. H., Nascimento, C. A. O. (2008). Application of Fluorescence Spectroscopy for Spectral Discrimination of Crude Oil Samples. *Brazilian J. Petrol. Gas*, *2*(2), 63–70.

Sparg, S. G., Light, M. E., Van Staden, J. (2004). Biological activities and distribution of plant saponins. *J. Ethnopharm.*, *94*(2–3), 219–243.

Strauss, A., Spengel, S. M., Schaffner, W. (1995). Saponins from root cultures of phytolacca acinosa. *Phytochem.*, *38*(4), 861–865.

Suga, Y., Maruyama, Y., Kawanashi, S., Shoji, J. (1978). Studies on the constituents of phytolaccaceous plants. I. On the structures of phytolaccasaponin B, E and G from the roots of Phytolacca americana L. *Chem. Pharm. Bulletin*, *26*(2), 520–525.

Vicente, G., Molina, S., Gonzalez-Vallinas, M., Garcia-Risco, M., Fornari, T., Reglero, G., Molina, A. (2013). Supercritical rosemary extracts, their antioxidant activity and effect on hepatic tumor progression. *J. Supercritical Fluids.* 79, 101-108.

Wang, L., Bai, L., Nagasawa, T., Hasegawa, T., Yang, X., Sakai, J., Bai, Y., Kataoka, T., Oka, S. (2008). Bioactive triterpene saponins from the roots of Phytolacca americana. *J. Nat. Prod.* 71(1), 35-40.

Woo, W. s. (1974). Steroids and pentacyclic triterperpenoids from *Phytolacca americana*. *Phytochem.*, *13*(1973), 2887–2889.

Yalavarthi, C., Thiruvengadarajan, V. S. (2013). A review on identification strategy of phyto constituents present in herbal plants. *Int. J. Res. Pharm. Sci.* , *4*(2), 123–140.

Yesil-Celiktas, O., Sevimli, C., Bedir, E., Vardar-Sukan, F. (2010). Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. *Plant Foods for Hum. Nutr.* 65(2), 158-163.

Zealand, N. (1975). *Phytolacca americana* (*Phytolaccaceae*) The plant Plant parts used Constituents of the root, (9), 1–5.

Zou, Y., Kim, D., Yagi, M., Yamasaki, Y., Kurita, J., Iida, T., Oda, T. (2013). Application of LDH-release assay to cellular-level evaluation of the toxic potential of harmful algal species. *Biosci.Biotechnol. Biochem.*, *77*(2), 345–352.